RUAN FELIPE VIEIRA MEDRANO

Remediation of the p53/Arf and interferon-beta pathways as a cancer immunotherapy strategy: a gene transfer approach

Thesis presented to the Faculdade de Medicina da Universidade de São Paulo to obtain the degree of Doctor in Science

Oncology Program

Supervisor: Prof. Dr. Bryan Eric Strauss

São Paulo

2017

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Remediação das vias p53/Arf e interferon-beta como uma estratégia de imunoterapia do câncer: uma abordagem de transferência gênica

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

Programa de Oncologia

Orientador: Prof. Dr. Bryan Eric Strauss

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To my colleagues from the viral vector laboratory and to my supervisor Dr. Bryan Strauss

”Science is a cooperative enterprise, spanning the generations. It's the passing of a torch from teacher, to student, to teacher. A community of minds reaching back to antiquity and forward to the stars “

Neil deGrasse Tyson

The doctoral journey can be considered as some sort of academic “adventure” full of ups and downs. And for this reason, I can`t help feeling very fortunate to have many people to thank.Each one on their own way, have contributed immensely to my development as a scientist.

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Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 3

Chapter 7

Medrano RFV. Remediação das vias p53 /Arf e Interferon-beta como uma estratégia de imunoterapia do câncer: uma abordagem de transferência gênica [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2017.

As células tumorais prosperam como consequência da capacidade de resistir aos mecanismos de morte celular e de evasão da vigilância imunológica. Nós propomos que, em cânceres que possuem o supressor de tumor p53 selvagem, a remediação de ambas dessas defesas pode ser promovida pela transferência genica combinada de vetores adenovirais portadores dos transgenes de $p19^{Arf}$ (proteína supressora de tumor, parceira funcional de p53) e de interferon-β (IFNβ, citocina imunomoduladora). De fato, em resultados anteriores, notamos que a transdução combinada (p19Arf/IFNβ), mas não os tratamentos individuais, em células de melanoma murino B16F10 resulta em aumento massivo de morte celular. Porém a capacidade destas células em processo de morte de desencadear imunidade antitumoral não foi analisada. Nesta tese e em estudos complementares, buscamos investigar os mecanismos moleculares de morte celular envolvidos na resposta imune estimulada por p19Arf/IFNβ e explorar sua aplicação como imunoterapia do câncer. Inicialmente, em modelo de vacinação profilática, revelamos que o tratamento combinado em células B16F10 promove a expressão de IL-15, ULBP1, dos receptores de morte FAS/APO1 e KILLER/DR5, assim como uma resposta de células natural killer que rejeitam estas células tratadas quando inoculadas em camundongos imunocompetentes singênicos. Após desafio tumoral no flanco oposto, a progressão desses tumores foi fortemente reduzida devido ao engajamento de linfócitos T $CD4^+$ e $CD8^+$, que apresentaram produção aumentada das citocinas IFN-γ e TNF-α e medeiam proteção antitumoral de longo prazo. Em seguida, explorando um contexto de imunização diferente, a transferência de gênica *in situ* foi realizada em carcinoma heterotópico de pulmão e exibiu proteção significativa contra um desafio tumoral secundário, apenas quando o tumor primário foi tratado com p19Arf/IFNβ. Análise de transcriptoma destes tumores indicou uma assinatura quimiotáxica de neutrófilos e linfócitos T $CD8^+$ através das quimiocinas CCL3, CXCL3 e da IL-1β. Em apoio destas observações, análises mecanicistas *in vitro* revelaram que células tratadas com p19Arf/IFNβ ativam programas apoptóticos de p53 e antivirais de IFNβ, enquanto sucumbem a um processo de morte por necroptose que também libera moléculas de morte celular imunogênica (MCI), calreticulina, ATP e HMGB1. No entanto, procurando potencializar ainda mais o benefício terapêutico dos nossos vetores, exploramos sua associação com o quimioterápico imunogênico doxorrubicina (Dox), que também é indutor de MCI. E nesta associação, percebemos que a Dox aumenta não apenas os níveis de morte celular, mas também a imunogenicidade das células tratadas, proporcionando em um modelo de vacina terapêutica, um controle tumoral superior em camundongos que já portavam antes da vacinação tumores B16F10 ou MCA205. Além disso, a associação *in situ* destas terapias restaurou a eficácia de uma dose sub-terapêutica de Dox, que em contraste com sua dose terapêutica, não prejudica a função cardíaca. Finalmente, também exploramos a associação com o bloqueio dos pontos de controle imunológicos PD-1 ou CTLA-4, que no modelo de vacina terapêutica, sua associação induziu maior rejeição completa de tumores B16F10. Em conclusão, aqui apresentamos evidências sobre a capacidade da combinação p19Arf/IFNβ de induzir morte celular e estimulação imunológica. E ressaltamos seu potencial como uma estratégia de imunoterapia do câncer.

Descritores: Melanoma experimental; Neoplasias; Vacinas anticâncer; Interferon tipo I; Proteína supressora de tumor p53; Morte celular.

Medrano RFV. Remediation of the p53/Arf and Interferon-beta pathways as a cancer immunotherapy strategy: a gene transfer approach [Thesis]. São Paulo:" Faculdade de Medicina, Universidade de São Paulo"; 2017.

Cancer cells thrive as a consequence of resisting cell death mechanisms and escaping from immune surveillance. We propose that, in cancers that harbor the wild-type tumor suppressor p53, remediation of both of these defenses can be achieved by harnessing the adenoviral vector mediated gene transfer of $p19^{Arf}$ (tumor suppressor protein, p53 functional partner) together with interferon-β (IFNβ, immunomodulatory cytokine). Indeed, in our initial observations, it was noticed that combined-transduction (p19Arf/IFNβ), but not the individual treatments, of B16F10 mouse melanoma cells results in massive cell death levels. Yet, the capability of these dying cells to unleash antitumor immunity was not investigated. Here in this thesis and in complementary studies, we sought to investigate the molecular mechanisms of cell death involved in the p19Arf/IFNβ immune stimulation and explore its potential as a mediator of cancer immunotherapy. First, in a prophylactic B16F10 vaccine model, we revealed that the dual treatment led to the up-regulation of IL-15, ULBP1, FAS/APO1 and KILLER/DR5 death receptors, plus a natural killer cell response that completely rejects treated cells when inoculated in syngeneic immunocompetent mice. Whereas, upon a contralateral tumor challenge, progression was strongly reduced by engaging both $CD4^+$ and $CD8^+$ T cells, which displayed augmented production of IFN-γ and TNF-α cytokines and provided long term antitumor protection. Next, exploring different immunization context, *in situ* gene transfer in a heterotopic lung carcinoma exhibited significant protection against a secondary tumor challenge only when the primary tumor was treated with p19Arf/IFNβ. Transcriptome analysis of these treated tumors indicated a chemotaxic signature of neutrophils and $CD8^+$ T cells with the involvement of CCL3, CXCL3 chemokines and IL-1β. Moreover, in support of this evidence, mechanistic *in vitro* studies revealed that p19Arf/IFNβ treated cells reactivate p53 apoptotic and IFNβ antiviral programs, while succumbing to a necroptosis cell death processes that also releases immunogenic cell death (ICD) molecules, calreticulin, ATP and HMGB1. Yet, aiming to potentiate therapeutic benefit of our vectors, we explored their association with doxorubicin (Dox) immunogenic chemotherapy, which is also an inducer of ICD. And in this setting, this association with Dox enhances not only cell death levels but also immunogenicity of treated cells, providing superior tumor control in a therapeutic vaccine model, where mice were already bearing B16F10 tumors or MCA205 sarcomas before vaccination. Moreover, associated use of these therapies *in situ* rescued efficacy of a sub-therapeutic dose of Dox, which in contrast to its therapeutic dose, does not impair cardiac function. Finally, we also evaluated the association with PD-1 or CTLA-4 checkpoint blockade immunotherapy, which in the therapeutic vaccine model induced full tumor rejection in a greater number of mice. In sum, here we provide compelling evidence for the ability of the p19Arf/IFNβ combined gene transfer to promote cell death and immunogenic stimuli and underscored its potential to be applied as a cancer immunotherapy strategy.

Descriptors: Melanoma experimental; Neoplasms; Cancer vaccines; Interferon type I; Tumor suppressor protein p53; Cell death.

1

Introduction and outline of the thesis

.

INTRODUCTION

"In animals…genetic changes must be common and a proportion…will represent a step towards malignancy…there should be some mechanism for eliminating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character." Burnet - The concept of immunological surveillance, 1970 (1).

This concept entails that the immune system works as an effective barrier against transformation of cancer cells and, although it was not an easy road, mounting evidence gathered throughout the years have not only supported this antitumoral role of immunity, but also demonstrated that the immune system from a cancer patient can be successfully exploited for therapeutic purposes, translating into significant and long lasting survival benefits (2).

Yet, the relationship between the immune system and cancer is far more complex. Since during the multistep development of neoplastic disease, malignant cells acquire the capability to resist cell death stimuli, deregulate the cell cycle machinery, activate a metastastic program and, through the establishment of an immune suppressive tumor microenvironment, the ability to evade from immune surveillance as well as hamper effectiveness of therapeutically elicited responses (3). Characteristics such as those are now well appreciated as the "Hallmarks of Cancer" and this progressive loss of intrinsic and extrinsic anti-tumor barriers has important implications for the conceptual ground on which this thesis is based.

Indeed, under the light of a therapeutic perspective, one could hypothesize that re-establishing the ability of cancer to succumb to cell death and to activate an immune response should be an appealing strategy. Take for example the treatment of malignant melanoma, which, due to its marked ability to rapidly metastasize, is considered to be one of the deadliest forms of cancer, but on the other hand, displays two features that we intend to take advantage of.

First, in contrast to other types of malignancies, melanoma retains the tumor suppressor protein p53 in its wild-type form in 90% of cases, although its apoptotic program is functionally disabled in part by the over expression of its negative regulator - mouse double minute 2 homolog (MDM2), seen in 56% of cases (4), or loss of its key functional partner - $p14^{ARF}$ (alternate reading frame, $p19^{Arf}$ in the mouse), observed in around 50% of cases (5). *Second*, melanomas have a high mutation and tumor neoantigen burden when compared to other tumor types (6), and so is endowed with a superior immunogenic potential that can be successfully targeted by cancer

immunotherapies, as evidenced by the clinical success of CTLA-4 and PD-1 checkpoint blockade.

Still on the $P14^{ARF}$ note, it is worth pointing out that its expression is induced by different forms of oncogenic stress (e.g, c-MYC, E1A and E2F1) and in its canonical mechanism of action, functions by preventing the MDM2 mediated degradation of p53, enabling p53 to trigger a different array of cellular responses, including cell cycle arrest, authophagy and apoptosis (7, 8). And that the $p14^{ARF}$ locus (known as *CDKN2A*) is located at human chromosome 9p21 that also harbors the interferon-beta (IFN-β) gene, a pivotal pleiotropic cytokine known for its antiviral effects as well as for its ability to strongly modulate innate and adaptive immune system compartments (9). But perhaps, even more interesting is the observation that homozygous deletions within chromosome band 9p21 have been reported in melanoma patients (10, 11), yet again highlighting the importance of cancer cells' subversion of cell death and immunological mechanisms in order to thrive even when confronted with the host response.

In fact, there is evidence for cooperation between the p53/Arf and IFN-β pathways. For example, upon exposure to type I IFNs (IFN-α/β), an Interferon-sensitive response element (ISRE) present in the p53 promoter has been shown to mediate the increase in p53 levels, culminating in the activation of pro-apoptotic p53 target genes (e.g., Puma, Bax) and augmenting antiviral response of infected cells by potentiating apoptosis (12, 13). Signaling trough IFN-regulatory factor 5 (IRF-5) and IRF-9, which are transcription factors with an essential role in mediating antiviral defense mechanisms by IFNs, have also been identified as direct targets of p53 (14, 15). However, in a cancer setting, one report suggests that cytostatic and apoptotic effects in response to IFN-α/β actually require a $p14^{ARF}$ pathway that is p53-independent (16).

Whereas these studies point out the intersection between the p53/Arf and type I IFN pathways, the cooperation between them has not been exploited as a therapeutic strategy in cancer. And so, aiming to remediate cell death mechanisms as well as antitumor immunity of melanoma, we have harnessed the p53/Arf and IFN-β pathways and developed a set of unique adenoviral vectors that encode the $p19^{Arf}$ and murine IFN-β cDNAs under the control of a p53 responsive synthetic promoter, called PGTxβ (PG) (17). The PG system was previously shown to direct superior transgene expression in comparison with traditional constitutive promoters (18) and to achieve an autoregulatory feedback mechanism when the PG vector encodes p53 itself (19) or alternatively, by targeting endogenous p53 upon reposition of $p19^{Arf}$ (20). We propose that the adenovirus mediated gene transfer of these transgenes to tumors that harbor

wild type p53 has the potential to create interplay between (i) transgene control, (ii) p53/Arf apoptotic stimuli and (iii) an IFN-β antiviral immunomodulatory context that ultimately comes together in order to unleash cell death in addition to evoking the immune system to attack the tumor.

Accordingly, in our initial observation, we noticed that the co-transduction of B16 mouse melanoma cells with the $p19^{Arf}$ and IFN-β adenoviral vectors, not the individual treatment, resulted in a synergic enhancement of cell death *in vitro* as well as in an *in vivo* model of gene therapy, where these vectors were directly injected into subcutaneous (s.c) tumors, markedly reducing tumor progression and increasing survival (17). Although these results we considered as promising, a critical question regarding the $p19^{Arf}$ and IFN-β combination was not investigated: could the cooperation between the p53/Arf and IFN-β pathways be applied as a cancer immunotherapy strategy?

The scope of this thesis is to address this question. Since use of IFN-β is already recognized for mediating effective antitumor immunity, it was critical for us to investigate how adding $p19^{Arf}$ would impact the immune response and if there was an actual therapeutic application for doing so. To this end, we have developed a prophylactic cancer vaccine model in which dying B16 cells (i.e., during the cell death process), treated *ex vivo* with the $p19^{Arf}$ and IFN-β combination, were applied as the vaccine agent in the left flank of immune competent syngeneic C57BL/6 mice. And seven days after immunization, naive fresh B16 cells were injected in the opposite side, acting as the tumor challenge and providing a readout of the immune protection provoked by the vaccination, where tumor progression is expected to be reduced in comparison to control groups.

At the time we proposed this model, the current point of view dictated that apoptotic and necrotic dying cells have different immunogenic potentials, being the first considered as tolerant and the latter considered as immunogenic due the release of immunogenic danger associated molecules (DAMPS) that end up working as natural adjuvants for antigen presenting cells (APCs) to achieve optimal maturation status. Although this dichotomized dogma is no longer accepted, owing to the emergence of the immunogenic cell death (ICD) concept, our hypothesis was that if $p19^{Arf}$ and IFN-β gene transfer were to provide an additional immunomodulatory stimulus, it would come from DAMPS released through the cell death process, since treatment with IFN-β alone does not kill tumors cells efficiently and may have limited ability to provide adjutancy.

DESCRIPTIVE OUTLINEOF THE THESIS

Before dwelling on the experimental data, in **Chapter 2** we present a review manuscript that provides theoretical background on how emerging targets in cancer immunity are setting the framework for the next era in immunotherapies. The review highlights the role of key cellular and molecular immune components as well as the development of immune evasion and acquired resistance mechanisms.

In **Chapter 3**, we present a review published in Oncotarget that discusses the central role that type I IFNs have in mediating tumor immune system interactions and the applications of harnessing their antitumor and immunomodulatory functions in cancer therapy.

In **Chapter 4**, we describe the results obtained with the vaccine model, which were published in Cancer Immunology and Immunotherapy, and indicates a remarkable antitumor protection effect that involves the participation of natural killer (NK) cells and is dependent on both $CD4^+$ and $CD8^+$ T cells.

In **Chapter 5,** in a study published in Translational Oncology, we aimed to uncover evidence that supports the results obtained with the vaccine model by exploring a different immunization context that consisted on the *in situ* treatment of tumors with the $p19^{Arf}$ and IFN-β adenoviral vectors. Our hypothesis postulated that immunization directly into the tumor mass would be more challenging for an antitumor immune response to be orchestrated and so could reveal the $p19^{Arf}$ and IFN-β immune benefit over IFN-β single treatment.

In **Chapter 6**, in a work published in Cell Death Discovery, we provide mechanistic insight on how the $p19^{Arf}$ and IFN-β combination unleashes cell death and secretes *bona fide* mediators of ICD.

In **Chapter 7**, we show data obtained from an ongoing project that aims to potentiate therapeutic outcome of the $p19^{Arf}$ and IFN-β gene transfer by exploring its combination with other immunotherapy strategies, Doxorubicin chemotherapy (i.e., a standard of care treatment modality and also an inducer of ICD) or CTLA-4 and PD-1 checkpoint blockade immunotherapy.

In continuation, **on Chapter 8** we provide a general discussion of the main evidence gathered throughout the aforementioned studies and ponder on how potential applications of $p19^{Arf}$ and IFN-β gene transfer fit in the current cancer immunotherapy scenario. Moreover, we also present the final considerations of this thesis.

Importantly, with the intent of being transparent to the thesis committee on my actual contributions to these studies, a detailed summary of each author's contributions is presented at the end of each chapter.

Additionally, in **Attachments A and B**, we provide other articles related to the body of work presented in this thesis, a invited research highlight article and a point of view article, that summarize our findings and feature our working hypothesis model on the immunotherapeutic cycle induced upon treatment with our vectors.

Lastly, in **Appendix A**, we describe the results obtained during the internship (November, 2015 to October, 2016) in the laboratory of Dr. Robert Schreiber, Department of Immunology and Pathology, Washington University in St. Louis, USA. The objective of this internship was to optimize a synthetic long peptide (SLP) neoantigen based vaccine and also to develop other vaccine platforms as means to achieve a more efficient immunization. Given that the use neoantigen vaccines represents the next frontier in cancer immunotherapy and, on a more general note, that the goal of harnessing the immune system to fight cancer is the same as the project developed in this thesis, we consider that presenting this results to be important for the thesis committee.

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2

Emerging targets in cancer immunity: bringing immunotherapies to its next era

Ruan F. V. Medrano, José A. M. Barburto, Roger Chammas and Bryan E. Strauss

Review manuscript

ABSTRACT

Modern cancer immunotherapy is enjoying of an exceptional clinical success and as the field moves steadily forward, emerging immunotherapeutic approaches are poised to target key cellular and molecular components of the immune system, of which neoantigen based vaccines are proposed to hit cancer immunity right in its core. Yet, the development of primary and acquired resistance is showing to be major obstacle for sustaining therapeutic responses in a significant portion of patients. Thus, in this review we intend to discuss how combinatorial strategies, identification of predictive biomarkers and immune monitoring methods are expected to circumvent these obstacles and pave the way for the field to enter in its next era.

INTRODUCTION – A BRIEF TIMELINE OF CANCER IMMUNOTHERAPY

Unveiling the nature of tumor immunity was a fundamental step towards the development of modern cancer immunotherapy. Accordingly, despite suggestive evidence with Coley's toxin in 1893, which applied bacterial products to elicit inflammatory responses, (1) even acknowledging that the immune system could detect and eliminate transformed cells was still a contentious issue at the end of the last century (2, 3). In fact, it was only in 2001 that the immune surveillance theory conjointly proposed by Ehrlich, Burnet and Thomas could be experimentally demonstrated, as immunodeficient $Rag2^{-/-}$ mice (lacking T, B and NKT cells) were reported to present an increased incidence of 3-methylcholanthrene (MCA) induced sarcomas in comparison to wild-type immunocompetent counterparts. Moreover, it was observed that tumors originating in the $Rag2^{-/-}$ background (i.e., absence of adaptive immunological pressure) were shown to be highly immunogenic, since 40% of them were rejected in a T lymphocyte dependent process when transplanted into naïve immunocompetent syngeneic hosts. In contrast, to tumors obtained from immunocompetent mice that grew progressively in all of the new hosts and as such, displayed a reduced immunogenic phenotype (4).

Through this elegant set of experiments and other subsequent studies, Schreiber have not only demonstrated that the immune system can indeed act as an effective extrinsic tumor-suppressor barrier, eliminating transformed cells, but also, that immunogenic cancer cells, kept in equilibrium state, can be edited out from the tumor mass and replaced by less immunogenic variants, which eventually escape from adaptive immunity and participate in the establishment of an immunosuppressive microenvironment that jeopardizers effective anticancer responses. This dynamic phenomenon has been termed "the cancer immunoediting hypothesis" (5, 6) and along with the work of many other groups, provided compelling evidence that evasion of immune attack should be recognized as one of the "Hallmarks of Cancer" (7).

However, initial attempts to harness the immune system came from the demonstration, in 1985, that administration of recombinant interleukin-2 (IL-2), a cytokine critical for T cell proliferation, provided a durable response in some melanoma patients (8) and, in 1991, the identification of the first human gene that encoded a tumor-associated antigen (named MAGEA1) (9). Such early approaches were mainly designed to promote inflammation in order to stimulate T cell immunity against shared antigens expressed on tumors, but did not properly address immune evasion

mechanisms employed by cancer cells. Indeed, even though infusion of high dose IL-2 or Interferon-α (IFN-α) was shortly after approved by the US Food and Drug Administration (FDA), durable responses were accompanied by severe adverse effects and were limited to a small fraction of patients (6 to 10%) in the case of IL-2 (10) or for adjuvant IFN-α treatment(11). Along the same lines, even with the identification of other tumor-associated antigens (12), cancer/testis antigens (e.g., NY-ESO-1) and development of different vaccine platforms (e.g., peptides and DNA vaccines), benefits were considered far too modest, failing to show objective clinical responses (13-15). For example, in the first of its kind FDA approved treatment, the autologous dendritic cell vaccine Sipuleucel-T for castration-resistant prostate cancer, which is based on the delivery of prostatic acid phosphatase antigen (PSA) along with granulocyte macrophage colony-stimulating factor (GM-CSF), only provided 4 months of survival increment when compared to placebo (16).

Concomitantly with these frustrating attempts, it was already known that T cell priming required dendritic cells (DCs) to provide co-stimulation signals through B7 molecules (B7-1 and B7-2, known also as CD80 and CD86, respectively) to their ligand (CD28) present on T cells (17). Yet, evidence also pointed for the induction of parallel activating or inhibitory mechanisms that, as it is now understood, are responsible for regulating the amplitude of the immune response, and thus referred as immunological checkpoints (18). Among these, the inhibitory program mediated by cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) was just being uncovered, a receptor that is rapidly expressed upon T cell activation and attenuates CD28 co-stimulation by also engaging on B7 molecules, but with a much superior avidity than CD28 (18, 19). And, in 1996, supporting the opposing roles of CD28 and CTLA-4 on T cell function, Alison provided seminal proof of principle regarding the targeting of immune checkpoints in cancer therapy, as anti-tumor T cell responses in tumor bearing mice were impaired by the antibody (Ab) mediated blockade of CD28, but profoundly enhanced upon blockade of CTLA-4, leading to tumor rejection and long term immune protection (20).

This and other subsequent observations (21, 22), especially its unprecedented clinical success, provided undeniable arguments about the potential of checkpoint blockade immunotherapy to release T cells from their regulatory brakes, thus representing a novel and more broad means of targeting the immune system that rejuvenated the field. In light of its 20% durable response rate, the anti-CTLA-4 humanized monoclonal Ab (Ipilimumab) was approved by the FDA in 2011 for the treatment of metastatic melanoma (23-25) and paved the way for the blockade of

another inhibitory checkpoint, the programmed death receptor-1 (PD-1) pathway, which targeted by different FDA approved Abs (e.g., Nivolumab, Pembrolizumab and Atezolizumab) has also obtained striking clinical results (20 to 50% durable response) across a range of different cancers, including advanced melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), bladder and others (26-32). Also during the decade of 2010, increasing the cancer immunotherapy momentum, adoptive cell transfer (ACT) of autologous *ex vivo* expanded T lymphocytes, genetically engineered or chimeric antigen receptor (CAR) T cells were demonstrated to mediate dramatic regression of different tumors, especially lymphoma and leukemia (33-35).

Furthermore, clinical evidence showing that CTLA-4 and PD-1 blockade could be synergistically combined, obtaining a tremendous 50% objective response rate in melanoma (36, 37), 40% in RCC (38) and 30-31% for NSCLC (39, 40), indicated that these immune checkpoint pathways are non-redundant and that combinatorial approaches have the potential to increase therapeutic efficacy. However, since regulatory pathways are being modulated, immunotoxicity and autoimmunity emerge as the Achille's heel for combinatorial strategies (41). And, a considerable fraction of cancer patients still fail to benefit, while others, after a period of response, develop acquired resistance (42). Indeed, advanced methods for immunomonitoring and identifying biomarkers that predict clinical outcome are expected to bring the field into a personalized era with greater efficacy and safety (43). Among those methods, recent technological innovations shed light on the role that cancer neoantigens play in cancer immunity and offer an attractive opportunity for the development of next-generation immunotherapies (44). Therefore, in this review, we will discuss how these current concepts are setting the foundation for emerging immunotherapeutic strategies to achieve the full potential of cancer immunity.

THERAPEUTIC TARGETS WITHIN THE CANCER-IMMUNITY CYCLE

As conceived by Chen and Mellman, the cancer-immunity cycle encompasses a series of tightly regulated steps that aim to promote an effector T cell mediated response as means to eradicate the tumor - while discerning self-antigens from non self-antigens (i.e., tumor antigens) present on the major histocompatibility complex (MHC) class I molecules of cancer cells and restraining development of autoimmunity. Therapies that interfere with the regulatory factors in these anti-tumor immune functions can either start a *de novo* T cell attack or re-invigorate an already pre-existing response, but with a more broad and potent therapeutic potential. Yet, on the other hand, due to immune

escape mechanisms and acquired resistance, cancer cells can inhibit the propagation of this cycle and hamper its efficacy (45).

Taking these concepts into consideration, in this section we intend to deepen our understating on how immune-based therapies can target the cancer immunity. *First,* we will delve into key cellular and molecular components that can be targeted in therapeutically elicited responses; and *secondly*, we discuss development of immune evasion and resistance mechanisms.

Natural killer cell based immunotherapies

Innate and adaptive immunity cooperate to eliminate tumors (46) and among the innate constituents, despite the increasing in recognition role of other innate lymphoid cell types (ILCs)(47), blood circulating Natural Killer (NK) cells are endowed with the ability to spontaneously kill tumor cells due their high cytotoxic capacity, which is mainly meditated by exocytosis of granules containing granzyme B and perforin (48, 49). Additionally, NKs can also positively influence maturation and antigen presenting functions of DCs(50-52), by producing inflammatory cytokines, such as tumor necrosis factor (TNF) and IFN-γ, which NKs are considered to be a major producer (48, 49). Thus, here we propose that augmenting therapeutic immune responses to cancer will be benefited by the modulation of NK cells antitumor functions **(Figure 1)** (53).

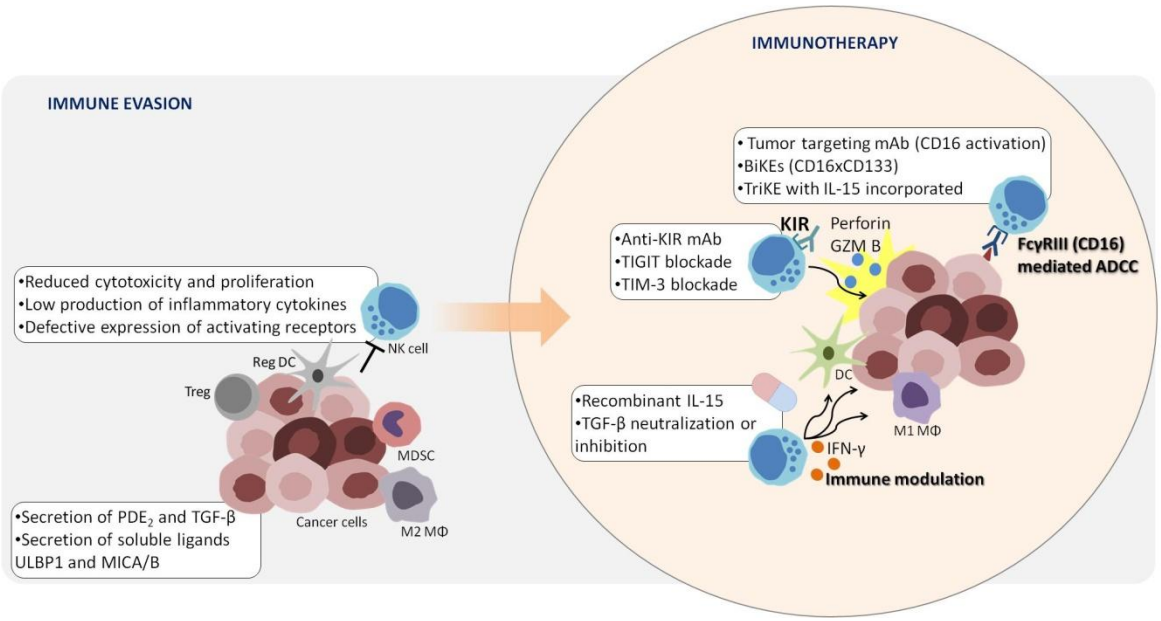

Figure 1. Targeting natural killer (NK) cells in cancer immunotherapy. The milieu of cells and molecules present within the tumor microenvironment displays of several pathways to evade from the antitumor functions of NK cells. Among these mechanisms, secretion of phosphodiesterase 2 (PDE_2), transforming growth factor-beta (TGF-β), as well as, soluble ligands for NKG2D activating NK cell receptor, as ULBP1 and MICA/B, can cause a dysfunctional NK cell state. Yet, NK cell based immunotherapies have the means to reestablish their functions. For example, blockade of inhibitory signals initiated by the binding of common KIR receptors, or TIM3 and TIGIT inhibitory checkpoints, can promote NK cells cytotoxicity, which is mediated by secretion of perforin and granzime-B (GZM-B)

granules. Moreover, NK cells can also be activated by opsonizing antibodies (Ab) upon cross-linking of the low-affinity IgG receptor FcγRIII (CD16), mediating antibody dependent cellular cytotoxicity (ADCC), which can be targeted by bispecific killer cell engagers (BiKE) and trispecific killer cell engagers (Trike) that successfully cross-link CD16. NK cells also display of immunomodulatory functions, where use of recombinant cytokines, such interleukin-15 (IL-15), and neutralization of TGF-β could allow NK cells to secrete interferon-gamma (IFN-γ) and favor maturation and antigen presenting functions of dendritic cells (DCs) and macrophages (mΦ). UL16 binding protein 1 (ULBP1); Major histocompatibility complex class I-related chain A/B (MICA/B); T cell immunoreceptor with Ig and ITIM domains (TIGIT); T-cell immunoglobulin and mucin-domain containing-3 (TIM-3).

The NK cells' mechanisms of action are resulting from complex interplay between several germ-line encoded activating and inhibitory receptors (54). Receptors for MHC-I molecules, named as Ly49 receptors in mice, killer immunoglobulin-like receptors (KIRs) in humans, confers inhibitory signals upon binding of self MHC-I, preventing attack on normal "healthy" cells of the host and, consequently, their "licensing" (i.e., activation) upon encounter with malignant or virus infect cells, that frequently down regulate the expression of MHC-I molecules, as resources to evade from adaptive immunity (55, 56). On the other hand, activation of NK cells comes from the recognition of stress-induced ligands on the surface of target cells that provide powerful stimulatory signals to their respective activating receptors, including NKp46, NKp30, NKp44, CD226 and the critical NKG2D, that binds several ligands (e.g., MICA/MICB and ULBP1) (57, 58). NK cells can also be activated by cytokines (e.g., IL-15, IL-18 and IFN-α), toll-like receptor (TLR) ligands and, notably, by opsonizing antibodies upon cross-linking of the low-affinity IgG receptor FcγRIII (CD16), mediating antibody dependent cellular cytotoxicity (ADCC) (54, 57, 58).

Numerous studies have shown that NK cells play a critical role in cancer immune surveillance (59), especially in the context of oncogene or DNA damage driven activation of intrinsic tumor suppressor pathways, such as the one mediated by p53 that can up-regulate NKG2D ligands ULBP1 and ULBP2 as well as chemokine recruiters of NK cells, for example CCL2 (60-62). Deficiency in the NK cell compartment in several models has been reported to result in superior growth of syngeneic transplanted tumors (57). In this regard, O'Sullivan, and collaborators have demonstrated that IFN-γ produced by NK cells prompted a tumoricidal M1 phenotype in macrophages and, importantly, absence of NK cells in the $RAG2^{-/-}$x $\gamma c^{-/-}$ mice results in a greater frequency of highly immunogenic regressor sarcomas, even when compared to their $RAG2^{-/-}$ counterpart (63). In a follow-up study, immune control exerted over tumor cells that remained in a "dormant" state was shown to depended on T cells, but not NK cells, supporting the notion that the main role for NK cells is eliminating nascent tumor cells (64).

Indeed, in established human tumors, infiltration of NK cells is found to be very poor and limited to the stroma region, (65, 66) and in terms of outcome correlations, represents a positive prognostic marker in diverse types of cancers (67-70). Yet, alterations in their functions have also been reported, such as reduction in cytotoxicity and proliferation, defective expression of activating receptors and inflammatory cytokines (71, 72). Tumor-associated immune cells, cytokines and metabolites, including, phosphodiesterase 2 (PDE_2), IL-10, and more critically, transforming growth factor-β (TGF-β), which recently was show to convert NKs cells into type 1 innate LCs devoid of cytotoxic function (73), and thus can greatly interfere with NK cell activity at the primary tumor site, favoring survival of pre-metastatic cancer cells that may then enter into the circulation (74-78). Tumor cells can also evade the NK mediated attack by secreting tumor-derived soluble ligands, including ULBP1 and MICA/B, which have been shown to down regulate NKG2D expression and associated with diminished cytolytic activity of NK and T cells (72, 79-81).

On base of this aforementioned evidence, a number of different NK-based therapies have been developed (a topic thoroughly reviewed in (82)). Here, we will focus on how endogenous NK cell responses may be modulated. And on this matter, IL-15, a cytokine that has an essential role on several aspects on NKs functions, is an appealing strategy. Indeed, in the recent first clinical trial using infusion of recombinant human IL-15 it was reported to be safe, increase proliferation and activation of NKs as well as clearance of lung lesions in metastatic melanoma patients (83). Additionally, an IL-15 super agonist Ab has also been reported to exert potent antitumor functions in different murine cancer models (84). Pharmaceutical inhibition of TGF-β is also considered an emerging, though non-specific, strategy for NK therapy (85).

Due to the expression of CD16, NK cells can recognize the variable fragment domain of scFV Abs and mediate ADCC, providing an opportunity for the design of novel scFv fusion proteins that enhance the antitumor activity of NK cells (86). A variety of bispecific killer cell engagers (BiKE) and trispecific killer cell engagers (Trike) that successfully cross-link CD16 have been described (87). For example, CD16xCD33 BiKE targeted primary leukemia samples (88) and was also shown to induce lysis of myeloid-derived suppressor cells (MDSC), which express CD33, potentially reversing immunosuppression of NK cells (89). In another example, a bispecific antibody against the stem-cell antigen CD133 was shown to target cancer stem-cells (CSC) (90) and more recently, engineering of an anti-CD133 Trike that incorporates IL-15 together with CD33 and CD16 was shown to provide a self-

sustaining mechanism via IL-15 signaling as well as improved NK cell performance against drug resistant CSCs (91).

Similar to the approach used with T cells, regulatory checkpoints of NK cells can be targeted by mAbs. The first demonstration of this strategy was performed by Romagné and collegues in 2009 and, by using a pan fully humanized antibody, called 1-7F9 (IPH2101), aimed to block inhibitory signals initiated by the binding of common KIR receptors (KIR2DL-1, KIR2DL-2 and KIR2DL-3) to HLA-C molecules (92). In this study, *in vitro* treatment with 1-7F9 mAb increased NK cell-mediated lysis of HLA-C-expressing tumor cells, without affecting normal peripheral blood mononuclear cells, and, *in vivo*, resulted in rejection of HLA-Cw3-positive target cells, thus providing a foundation for further clinical development (92). Yet clinical studies using IPH2101mAb failed to provide satisfactory therapeutic response as a single agent, although it was considered safe and well tolerated by patients (93, 94). Upon its combination with Lenalidomide, a derivative of Thalidomide used for multiple myeloma treatment, objective responses were observed, indicating that harnessing these checkpoints requires combination strategies (95). Other inhibitory receptors are emerging, including the targeting of NKG2A-CD94 (binds to the non-classical HLA class I molecule HLA-E) (96, 97), and the immunoglobulin family CD96 (known as TACTILE) and TIGIT (T cell Ig and ITIM domain), which both bind to nectin and nectin-like ligands overexpressed in some cancer types (98). Although only monalizumab (IPH2201), a blocking mAb to NKG2A, is currently being investigated in clinical trials (82, 85).

Identification of a novel inhibitory checkpoint called cytokine-inducible SH2-containing protein (CIS, encoded by *Cish*) was recently reported. By acting in a negative feedback loop, CIS is considered to be a critical physiological regulator of IL-15 signaling in NK cells, as deletion of the *Cish* gene renders NKs hypertensive to IL-15 stimulation (evidenced by increased JAK-STAT signaling, proliferation and cytotoxicity) (99). Accordingly, challenge of $Cish^{-/-}$ mice with metastatic tumor cells resulted in the formation of very few lung nodules as compared to WT $Cish^{+/+}$ mice, indicating the antitumor potential of targeting this checkpoint (99). In parallel, the combination of anti-CTLA-4 or anti-PD-1 along with NK checkpoint blockade, anti-CD96, was shown to further inhibit the formation of lung metastases, along with elevated IFNγ production and infiltration of NK cells (100). Thus, it is tempting to speculate that combining CIS inhibition with checkpoint blockade of CD96, IL-15 or PD-1 blockade could provide even more powerful antitumor protection. In another

study investigating inhibitory checkpoints that could be exploited to reverse NK cell dysfunction, T cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3), but not CTLA-4 or PD-1, was identified among the receptors whose expression was progressively increased on NK cells isolated from patients with metastatic melanoma and, remarkably, blockade of TIM-3 could reinvigorate exhausted NK cells and restore NK cell cytotoxicity, cytokine production and proliferation (101).

Dendritic cell based immunotherapies

Generation of tumor antigen-specific T cells is widely considered as a requirement for effective antitumor immunity (4, 64), a response that depends on the unique ability of DCs to endocytose dead cancer cells or cellular debris, transport captured tumor-associated antigens to peripheral lymph nodes (LN) and prime naïve T cell, by presenting processed antigens (peptides) loaded on MHC-I and MHC-II molecules to their cognate T cell receptor (TCR) on $CD8^+$ and $CD4^+$ T cells, respectively - while, also delivering co-stimulatory signals through surface CD80/CD86/CD40 molecules and secretion of cytokines, such as IL-12 and IFN-γ (102).

Among the different DCs subsets shown to perform this function, DCs driven by the transcriptional factor BAFT3, display the capacity to cross-present exogenously captured antigens on MHC-I in order to activate $CD8^+$ T cell responses and, as such, have a well appreciated role in cancer immunity (102, 103). Importantly, BAFT 3 driven DCs, express the X-C motif chemokine receptor 1 (XCR1), and encompass $CD8\alpha^+$ DCs, which are residents of lymphoid organs, such as spleen and LN, as well as $CD103^+$ cDCs, migratory and found in non-lymphoid organs (102, 103). In humans, $CD103^+$ DCs homologous are identified by the expression of CD141 (also known as BDCA3) (104).

Critical evidence concerning the role of $CD103^+$ DCs came from two studies. In the first, immunocompetent *Baft3*$^{-/-}$ mice, deficient in both $CD103^+$ and $CD8\alpha^+$ DCs compartments, were shown to be unable to reject highly immunogenic transplanted MCA induced sarcomas that spontaneously regresses when inoculated into wild type immunocompetent mice (105). In the second, $CD103^+$ DCs were required to achieve therapeutic benefit from PD-L1 checkpoint blockade, since *Baft3*$^{-/-}$ mice failed to respond (106). More recently, mechanistic insight was provided by Roberts and colleagues, which investigated different tumor associated APCs and observed that only $CD103^+$ could capture and transport intact antigens exclusively expressed in subcutaneous tumor cells back to the draining LN, while still being capable of drive

tumor specific $CD8^+$ T cell responses, thus evidencing their unique ability to cross-present antigens captured from the tumor. Notably, this antigen traffic process was mediated by the chemokine CCR7 axis (107).

Infiltration of tumor associated DCs (tDCs) has been observed in a diverse range of tumors, with clinical correlations remain somewhat ambiguous, being associated with both good and poor prognosis (108-110). Even so, presence of $CD141^+$ DCs was found to correlate with a superior outcome in melanoma patients (111). But, more recently, in early lung adenocarcinoma lesions it was observed that $CD141^+$ DC are significantly absent in comparison with non-lung adenocarcinoma tissue, whereas other DCs subsets were observed more frequently (112). Localization is also critical since DCs found in the tumor margins, rather than the infiltrating DCs, are thought to effectively cross-present tumor antigens and stably engage tumor-specific T cells (113). Accordingly, several tumor-derived factors can strongly impair DC functions, as observed in many studies showing that whilst the tumor progresses, DCs tend to exhibit a phenotype of low co-stimulatory molecule expression, inefficient antigen cross-presentation, expression of suppressive molecules and, can assume a tolerogenic phenotype (114-117). Of note, tumor supernatants containing high levels of vascular endothelial growth factor (VEGF) were found to significantly suppress secretion IL-12 by DCs (118), PGE2 induced arginase I mediated immunosuppression in DCs (119), IL-10 drove a STAT3 signature, and expression of S100A9 protein halted differentiation and maturation of DCs (120-122), which all together can contribute to the infiltration of immature monocytes to persist as a heterogeneous population MDSCs that can profoundly impair effector immune cell activity (123).

In light of these mechanisms of immune suppression, DC-based immunotherapies have mainly focused on *ex vivo* differentiation of DCs aiming to achieve optimal DC maturation and after antigen loading, once injected back into to the patient, elicit effective T cell priming (124). Although, significant development has been obtained with this appealing modality of vaccination and indeed is shown able to induce T cells responses against target antigens (125-127), as said before, clinical responses were limited to a small number of patients. Reasons for not achieving superior results are thought to include enrolment of patients with late-stage disease, use of DC vaccine without blocking immune checkpoints and, as will be discussed later, the type of tumor antigen used(124). Yet, it's important to stress that applications of *ex vivo* derived DCs and future development remains as a promising endeavor for the field and has been extensively reviewed elsewhere (128).

Therefore, here we will focus on *in vivo* strategies to modulate endogenous DCs, with the proposed advantage of harnessing tumor antigens that the DCs have already presented during prior spontaneous immune responses **(Figure 2)**. In a pioneering demonstration of targeting DCs *in vivo*, Steinman exploited the DEC205 surface receptor, which belongs to the C-type lectin receptor family and is recycled through late endosomal or lysosomal compartments, mediating antigen presentation (129). In mice, DEC205 expression is considered to be relatively restricted to mature DCs, including $CD8\alpha^+$ DCs. Thus, by coupling antigens to Abs against this DC receptor it would be feasible to specifically deliver antigens *in vivo*. Interestingly, antigen targeting of DEC205 without adjuvancy was found to induce regulatory T cells (Tregs), (129, 130). In contrast, delivery along with proper maturation stimuli leads to induction of both $CD4^+$ and $CD8^+$ T cell responses, as well as protective antitumor immunity (131).

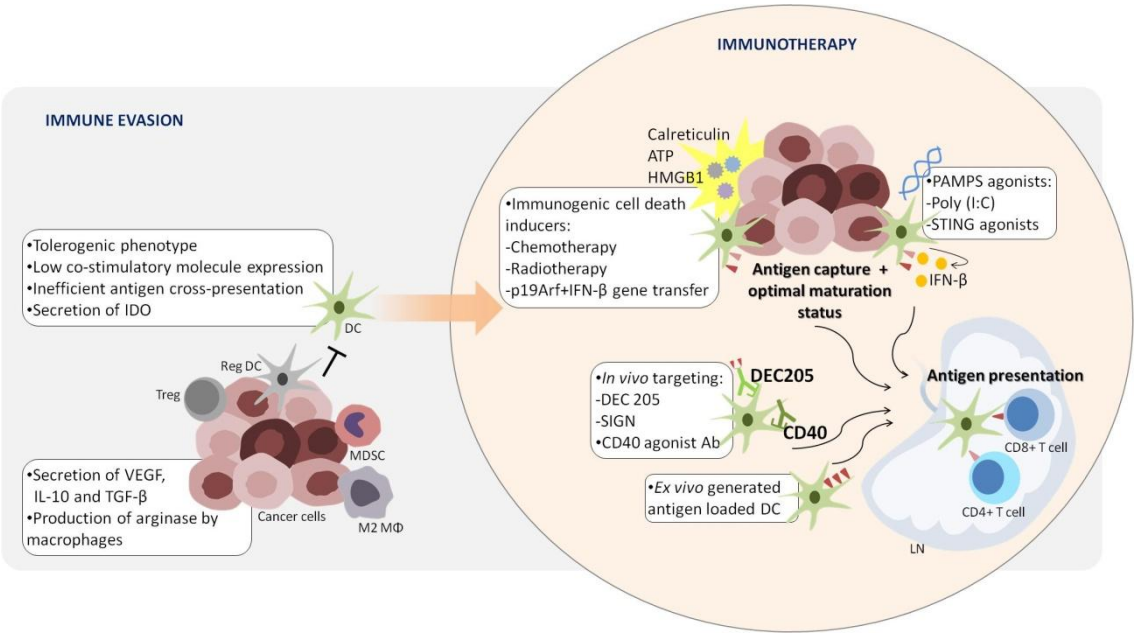

Figure 2. Targeting dendritic cells (DCs) in cancer immunotherapy. The components of the tumor microenvironment can greatly impact on DCs functions and among the main mechanism of immune evasion, secretion of vascular endothelial growth factor (VEGF), interleukin (IL-10) and transforming growth factor-beta (TGF-β), production of arginase-I by macrophages (mΦ) can, all together, halt maturation and differentiation of DCs, mediating to a tolerogenic phenotype, in which DCs act to suppress T cell immunity. However, immunomodulatory effects of DC-based immunotherapies have been shown to be able to change this scenario. For example, inducers of immunogenic cell death (ICD), such as doxorubicin chemotherapy and the adenoviral mediated gene transfer of $p19^{Arf}$ and interferon-β (IFN-β), can mediate the release of immunogenic DAMPS, such as calreticulin, ATP and HMGB1, to promote optimal DCs maturation. Maturation of DCs can also be achieved by the use of STING and TLR agonists that leads to the production of IFN-β. In this inflammatory context, tumor antigen capture should be followed by lymph node (LN) migration, where during T cell priming, mature DCs cross-present phagocytosed and processed antigens in MHC-I molecules to $CD8^+$ T cells and present in MHC-II molecules to $CD4^+$ T cells, while also providing co-stimulatory signals through their CD80, CD86, CD40 molecules. Alternatively, delivery of tumor antigens can also be performed *in vivo* by targeting distinct DCs receptors, like DEC205 and SIGN. Immune checkpoints related to DCs function can also be targeted, of which activation of CD40 via agonist CD40 mAbs are proposed to mimic the role of natural CD40L stimulation provided during $CD4^+$ T cells licensing. Lastly, *ex vivo* generated DCs can be fully matured and loaded with tumor antigens and drive a novel immunity cycle upon vaccination. pathogen-

derived molecular patterns (PAMPS); danger-associated molecular patterns (DAMPs); Indoleamine 2,3-dioxygenase (IDO); stimulator of interferon genes complex (STING); toll-like Receptors (TLR); major histocompatibility complex (MHC); DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin)..

Numerous other DC-targeting strategies have since been developed, for example targeting of c-type lectin receptors SIGN (132), DCIR (133, 134) or NK lectin group receptor-1 (DNGR-1/CLEC9) (135, 136), the latter of which was recently shown to be a plasmatic receptor for necrotic cells that is highly expressed in $CD8\alpha^+$ DCs and acts by directing necrotic cell cargo into a recycling endosomal compartment, favoring cross-presentation to $CD8^+$ T cells (137, 138). Even though the induction of tumor-specific T cells was observed in humans (139), clinical benefit remains to be defined, as multiple clinical trials are still ongoing (140). It is also tempting to speculate about the potential clinical benefits of XCR1, since this chemokine receptor is exclusively expressed on $CD141^+$DCs and has already been demonstrated to induce potent $CD8^+$ T cell cytotoxicity upon mAb targeting (141).

DC activation is induced trough the detection of pathogen-derived molecular patterns (PAMPS) by pattern recognition receptors (PRR), such as double-stranded RNA (dsRNA) from RNA viruses detected by TLR 3 and both cytosolic DNA and second messenger cyclic di-GMP24 from bacteria recognized by the stimulator of IFN genes (STING). Importantly, the TLR signaling cascade culminates in the activation of IFN-regulatory factor 3/7 (IRF3/7) or nuclear factor-κB (NF-κB) transcription factors and consequently, production of inflammatory cytokines as well as type I IFNs, more specifically, IFN-α and IFN-β (142).

IFN-α/β are pleiotropic cytokines that play a pivotal role in cancer immune interactions, which importantly, is temporally and functionally distinct from the role exerted by IFN-γ (the only type II IFN member, produced mainly by Th1 helper lymphocytes and NK cells) (143). Endogenous production of IFN-α/β by the host hematopoietic system was shown to be required for the prevention of growth by MCA-induced sarcomas and in tumor transplanting settings (144) And, specifically critical for $CD8\alpha^+$ DCs to cross-present antigens to $CD8^+$ T cells (145). Notably, type I IFN's immunomodulatory properties can increases cytotoxicity mediated by NK cells, cytotoxicity and survival of $CD8^+$ T cells. Additionally, activation of the JAK/STAT signaling cascade by type I IFNs also directly affects tumor cells by promoting growth arrest, cell death, and MHC-I up-regulation, which enhances cancers immunogenicity (146).

Based on these properties, different cancer therapies have been developed with the intent to promote type I IFNs in the tumor microenvironment, including gene therapy, which can transfer the IFN-α/β cDNA directly to tumor cells or to *ex vivo* modified DCs, recombinant proteins and innate inducers, such as TLR agonists (a subject we thoroughly reviewed elsewhere (147)). For example, Poly (I:C) and its more stable form Poly (IC:LC) targets TLR3 (148), Imiquimod and Resiquimod are TLR7/8 agonists (149) and several recently reported STING agonists have gained interest, like 2'-3'-cyclic GMP-AMP (cGAMP) (150), DMXAA (151), PC7A nanovaccine (152), cytosolic cyclic dinucleotides (CDNs) ligands (153), which by activating TBK1/IRF3 (interferon regulatory factor 3), NF-kB, and STAT6 signaling, were shown to unleash antitumor responses capable of causing regression of established tumors resistant to anti PD-1 blockade (153).

Yet, it is unlikely that these treatments will provide therapeutic benefit as single agents, since most of them are considered to act as adjuvants, leading to the use of combinatorial strategies that are expected to activate DCs in a synergistic manner. For example, recently, aiming to counteract the scarcity of $CD103^+$ DCs at the tumor site, Salom and colleagues showed that combined systemic administration of growth factor FMS-like tyrosine kinase 3 ligand (FLT3-L), a cytokine key for promoting commitment to the DC lineage as well as DC survival and proliferation, together with intratumoral injections of poly I:C expands and activates $CD103^+$ DC progenitors in the TME and, consequently, synergizing with anti-PD-L1 immunotherapy (106).

Similar to PAMPS, DCs can also be alerted by engaging danger-associated molecular patterns (DAMPs) that are produced upon cellular stress (e.g., heat shock proteins) or by dying cells throughout the induction of immunogenic cell death (ICD) in response to cancer therapy (142). ICD is a molecular process, in which dying tumor succumb the spatiotemporal release of calreticulin (154), ATP (155, 156), expression of CXCL1 induced by the autocrine secretion of IFN-β (induced by the binding of self RNA from dying cells to TLR3) (157) and high-mobility group box 1 (HMGB1) (158) in order to promote intratumoral recruitment and full maturation status of DCs and effective priming of $CD8^+$ T cells(159). And, although originally described as a cellular response to chemotherapy, several other activators of ICD have been discovered, each of which with a specific ability to elicit these *bona fide* ICD markers (160). Examples of such inducers include doxorubicin (161), radiotherapy (158), newcastle disease virus (NDV) oncolytic vector (162) and necroptotic cell death (163), and recent data from our lab shows that adenovirus mediated gene transfer of p19Arf (tumor suppressor protein,

p53 functional partner) in combination with IFN-β cytokine should be included among these strategies (164).

Indeed, in our initial observations, co-transduction, but not the individual treatment, of murine melanoma cells results in a synergic enhancement of cell death (165) and, as later explored in vaccine as well as *in situ* gene therapy models, a remarkable protective antitumor immune response mediated by NK cell, neutrophils and both $CD4^+$ and $CD8^+$ T cells (166, 167). Further analysis revealed that p19Arf/IFN-β gene transfer induced cell death was independent of caspase 3 activity, displays features of necropotisis (evidenced by receptor-interacting serine-threonine kinase 3, RIP3, and Tnfrsf1A up regulation) and releases ICD markers (calreticulin, ATP and HMGB1) (168). We have not yet evaluated the impact of our therapy on the APC compartment, yet immunotherapeutic applications of ICD involving maturation of DCs are gaining ground due to their high immunogenic potential. As exemplified in the first of two recent reports, ICD elicited by hypericin-based photodynamic therapy was used *ex vivo* to mature derived DCs and upon vaccination provoked T cell-driven rejection of high-grade gliomas (169). Whereas, in the second, prophylactic vaccination with necroptotic cancer cells was shown to lead to efficient anti-tumor immunity with the cross-priming and proliferation of $CD8^+$ T cells (170).

Finally, immune checkpoints related to DCs function can also be targeted therapeutically. CD40 is a co-stimulatory molecule that belongs to the TNF superfamily frequently present on APCs and is essential for differentiation of immature into fully mature DCs (171). Notably, activation of CD40 via its ligand CD40L expressed on $CD4^+$ helper T cells is thought to be a main mechanism of APC licensing, as activation of the CD40/CD40L pathway augments expression of co-stimulatory and MHC-I molecules, production of Th1 related cytokines and enhances T cell priming (171). Accordingly, immunotherapy with agonist CD40 mAbs were shown to synergize with chemotherapy in the treatment of established murine solid tumors (172) and to overcome resistance to PD-1 and CTLA-4 blockade in a pancreatic carcinoma model (173), thus indicating that agonist anti-CD40 mAbs are able to mimic the role of natural CD40L stimulation. Intriguingly, anti-CD40 tumor regression effect was also reported to induce the tumor infiltrating macrophages to deplete tumor stroma components and to assume tumoricidal phenotype which was independent of T cells (174). Phase I clinical trials with agonistic CD40 mAbs were associated with antitumor activity, where no cases of severe toxicities reported when used as a single agent (175) and in

combination with chemotherapy (176, 177). Combination strategies with PD-L1 checkpoint blockade are also expected to provide superior results (178).

Targeting T cell immunity

Next, reaching the effectors of cancer immunity, we will discuss how T cells, in particular activated cytotoxic $CD8^+$ T cells (i.e., cytotoxic T lymphocytes - CTLs) and th1 helper $CD4^+$ T cells, can be harnessed by cancer immunotherapy **(Figure 3)**.

In recent years, two distinct approaches have emerged as clear leaders, ACT and checkpoint blockade. ACT involves the use of naturally occurring autologous tumor infiltrating lymphocytes (TILs), which are collected from tumor samples, stimulated and expanded in the laboratory and then reintroduced in the patient to carry out tumor specific responses. The *ex vivo* manipulation of these cells creates an opportunity for enrichment of antigen-specific T cells (179, 180) or even for gene transfer modifications, resulting in genetically engineered or CAR T cells, before the cells are expanded and returned to the patient. CAR T cells targeting CD19 have been quite successfully applied for the eradication of B cell leukemias and FDA approval has been recommended (181), though fine tuning of this approach may reduce the occurrence of tumor lysis syndrome (182, 183). Progress related to ACT has been extensively reviewed elsewhere, including further description of the approach and clinical outcome (179).

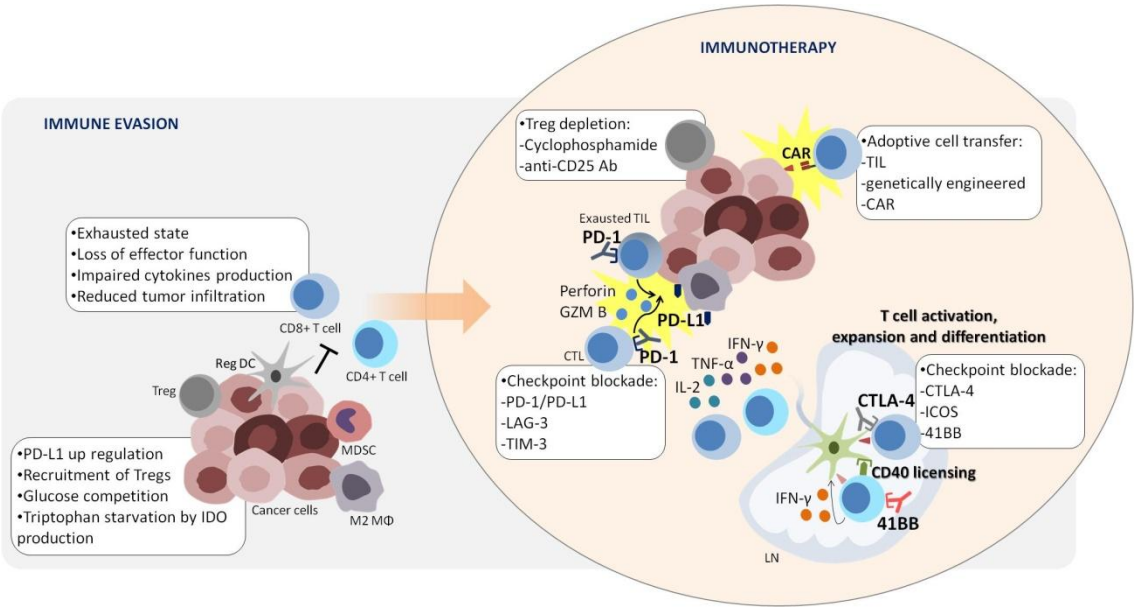

Figure 3. Targeting T cell immunity in cancer immunotherapy. Cancer cells employ of several mechanism to evade from the antitumor functions of T cells. Most notably, up regulation of programmed death-ligand 1 (PD-L1) by cancer cells and macrophages (mΦ), recruitment of regulatory T cells (Tregs) and tryptophan starvation by the enzyme IDO, induces an exhaustion phenotype in tumor infiltrating T cells (TIL), which presents impaired effector function with reduced production of cytokines. Yet, upon PD-1/PD-L1, LAG3 or TIM3 checkpoint blockade immunotherapy, exhausted $CD8^+$ T cells can have their cytotoxic functions reestablished, secreting perforin and granzyme B (GZM B) to induce cell death

of cancer cells. Alternatively, poly-functional $CD8^+$ and $CD4^+$ T cells can secrete interleukin-2 (IL-2), TNF-α and interferon-γ (IFN-γ) to sustain proliferation and function of TILs. Checkpoint blockade with anti-CTLA-4 monoclonal antibodies (Ab) or ICOS and 41BB agonists, can act during DC priming and augment T cell activation, differentiation and expansion. Moreover, depletion of Tregs with cyclophosphamide or anti-CD25 Abs can be employed to inhibit their suppressive role. And, in a more direct approach, adoptive cell transfer of TILs, genetically engineered and CAR-T cells can provide an opportunity to inject large amounts of antigen-specific T cells. Indoleamine-pyrrole 2,3-dioxygenase (IDO); Lymphocyte-activation gene 3 (LAG3); T-cell immunoglobulin and mucin-domain containing-3 (TIM-3); tumor necrosis factor-α (TNF-α); Inducible T-cell COStimulator (ICOS); Cytotoxic T-lymphocyte associated protein 4 (CTLA-4).

Alternatively, modulation of T cell immunity can be achieved by targeting its immunological checkpoints, including CTLA-4 and PD-1, as already mentioned in this review. In addition, other co-inhibitory receptors with potential clinical applications have been identified, including B- and T-lymphocyte attenuator-4 (BTLA-4), T cell immunoglobulin and mucin-containing molecule-3 (TIM3) and lymphocyte activation gene 3 (LAG3). Co-stimulatory receptors are also being intensively investigated, including CD28, inducible T-cell costimulator (ICOS) and members of the TNFR family - such as 4-1BB, CD27, OX-40 (184). The engagement of these T cell receptors to their cognate ligands expressed on APCs can take place in secondary lymphoid organs or in the periphery, as well, resulting in T cells activation, differentiation, proliferation and lead to effector and memory functions (184).

It is well established that tumors exploit certain of these checkpoints to escape elimination by the immune system. For example, in contrast to CTLA-4 whose ligand C80/86 is expressed on APCs and acts the stage of T-cell priming (185), the two ligands of PD-1, PDL-1 (B7-H1) and PD-L2 (B7-DC), are broadly expressed among different cell types, such as endothelial cells (186), fibroblasts (187) and notably, tumor cells (188). And as such, their role is to limit tissue damage in the periphery provoked by inflammatory immune responses since expression of PD-L1 is regulated by IFN-γ and type I IFNs, while PD-1 is induced when T cells become activated (i.e., in the effector phase) and declines after antigen clearance (189, 190). Binding of PD-1 to PD-L1 induces the recruitment of the tyrosine phosphatase SHP-2, leading to reduced phosphorylation of TCR signaling molecules, necessary for the induction of transcription factors Gata3, Tbx21 (T-bet), and Eomes, and secretion of IL-2, which consequently impairs T cell activation and proliferation (191). Yet, just recently, the T cell co-stimulatory receptor CD28 was identified as a critical target of SHP-2, as CD28 was shown to be desphosphorylated preferentially over the TCR, indicating that inhibition of CD28 signaling plays a key role in regulating T cell function (192). In parallel, Kamphorst and collaborators corroborated these findings by showing that CD28 is required for PD-1 immunotherapy to rescue $CD8^+$ T cells and that in the blood

of responders to PD-1 therapy, proliferating $CD8^+$ T cells were also $CD28^+$ (193). Moreover, the PD-1/PD-L1 program can also negatively affect IFN-γ production and cell survival(191). Interestingly, in the face of chronic antigen stimulation, for instance in the cancer or viral infection, PD-1 levels remain high and T cells undergo a state of exhaustion, which is defined by progressive loss of effector function, sustained expression of inhibitory receptors and a transcriptional state different from effector or memory T cells, and results in suboptimal control of viral infections (e.g., lymphocytic choriomeningitis virus, LCMV) and, importantly, to tumor immune escape (189, 194). Nonetheless, according to Schietinger and Greenburg, exhaustion should neither be considered as an unchanging, irreversible differentiation condition, nor as a state where T cells are unresponsive. Instead, exhaustion is an adaptive state of *hypo*responsiveness, which can be successfully "restored' by PD-1/PD-L1 checkpoint blockade immunotherapy (194). This implies a critical distinction between vaccine strategies, which promote a *de novo* immunity cycle, from the existence of a tumor-specific T cell repertoire that is an essential target for successful immune checkpoint blockade.

So far we have discussed aspects of dysfunctional tumor-specific T cells, but not the moment when they assume the unresponsive state. In a recent study, in order to gain further insight on this matter, an autochthonous liver cancer model with the induced expression of SV40 large T antigen revealed naïve and Ag experienced $CD8^+$ T cells from the beginning of the transformation process through the stages of tumor progression (195). Intriguingly, phonotypical and functional markers of exhaustion (i.e., PD-1, LAG3, and TIM3 and impaired cytokines production) were observed to be more profoundly expressed in the late stages of tumor progression, yet were already present at the initial phases, thus arguing that T cell dysfunction in the tumor can start even before tumor development and the establishment of the immunosuppressive TME. Moreover, by isolating cells from these different time points and using *ex vivo* PD-1 blockade, it was observed that $CD8^+$ T cells could have their effector function restored when obtained on day 8, whereas, if obtained on day 35, they entered a permanent, irreversible dysfunction state that was maintained even in the absence of antigen or dissociated from the tumor, indicating a narrow therapeutic window for the PD-1 checkpoint blockade.

Also working to understand the temporal dynamics of the PD-1/PD-L1 pathway, Noguchi and colleagues explored two closely related clones of MCA sarcoma, one that was highly immunogenic and spontaneously regresses in WT immunocompetent mice, and another that progresses in this context, but still preserves an immunogenic potential

that can be targeted by anti PD-1/PD-L1 immunotherapy (196). And, even though the immunogenic clone responded to IFN-γ by up-regulating PD-L1, its high immunogenicity was sufficient to circumvent the PD-L1 program, which could only mediate tumor escape if PD-L1 was expressed at supra-physiologic levels. However, CRISPR/Cas9 mediated deletion of PD-L1 was able to convert the progressive cell line into a regressor, indicating an inverse relationship between tumor antigenicity and the capacity of PD-L1 to promote tumor escape, as its expression was shown to play a role only in clones whose highly immunogenic antigens had been lost during tumor progression.

In this same last study, by studying the dynamics of PD-L1expression *in vivo*, it was observed that host cells CD45+ cells, more specifically macrophages, are the major contributors for PD-L1 levels within the TME, not the tumor cells(196). And, upon the blockade of IFN-γ, PD-L1 expression is strongly reduced on tumor cells, but still persists on host immune cells, indicating an alternative regulatory mechanism, not dependent of IFN-γ, is also involved. Remarkably, using the PD-L1 KO sarcoma, it was shown that PD-1 immunotherapy can function, even when the only source of PD-L1 is the host. Studies from other groups have also supported this notion that both tumor and host derived PD-L1 have non-redundant roles suppressing $CD8^+$ T cell cytotoxicity (197, 198) or even suggesting that PD-1/PD-L1 blockade works in a macrophage-dependent fashion(199), which as we are going to discuss later can impact the use of PD-L immunotherapy as first line treatment.

PD-1 blockade has been shown effective and its clinical application is enjoying an unprecedented success in the oncology field, inducing durable responses in tumors from different histologies, yet a significant fraction of patients either fail to respond or the therapeutic efficacy is low, or even after a period of response, relapse occurs (31). Distinct immunological mechanisms underlie these scenarios and as such will have different consequences for the clinic management of patients. Where efficacy was weak or not detected, the immune system was already unresponsive to therapy, characterized, as proposed by Sharma and colleagues, as primary resistance (42). In the case of relapse, immune attack was achieved, but not sustained in face of adaptive resistance mechanisms, defined as acquired resistance (42) **(Figure 4)**.

In fact, PD-1 immunotherapy has been suggested to depend on the levels of exhaustion, as demonstrated that reestablishment of effector function could be achieved in T cells with intermedium levels of PD-1, but not in terminally exhausted, with high levels of PD-1 (200). And as so, presence of a spontaneous $CD8^+$ T cell infiltrate is

clearly critical, which in inflamed tumors (i.e., figuratively speaking "hot tumors') evidence indicates to be mediated by a chemokine influx involving CXCR3 with its ligands CXCL9 and CXCL10(201) as well by a type I IFN signature to support DCs maturation(202). Alternatively, tumor intrinsic oncogene pathways have been show to mediate exclusion of T cells from the TME, but not macrophages, resulting in "cold tumors", refractory for immune based therapies(203). Of the pathways identified, activation of the WNT/β-catenin signalling pathway was demonstrated to inhibit CCL4 production by melanoma cells themselves, as a consequence of the transcriptional repressor ATF3, which ends up halting recruitment of $CD103^{+}$ DCc and preventing cross-priming of $CD8^{+}$ T cells(204). More recently, decreased T-cell infiltration and resistance to T cell-mediated cell death were also related to loss of phosphatase and tensin homologue (PTEN) and PI3K-AKT activation (205) and by epigenetic silencing of Th1 chemokines CXCL9 and CXCL10 (206).

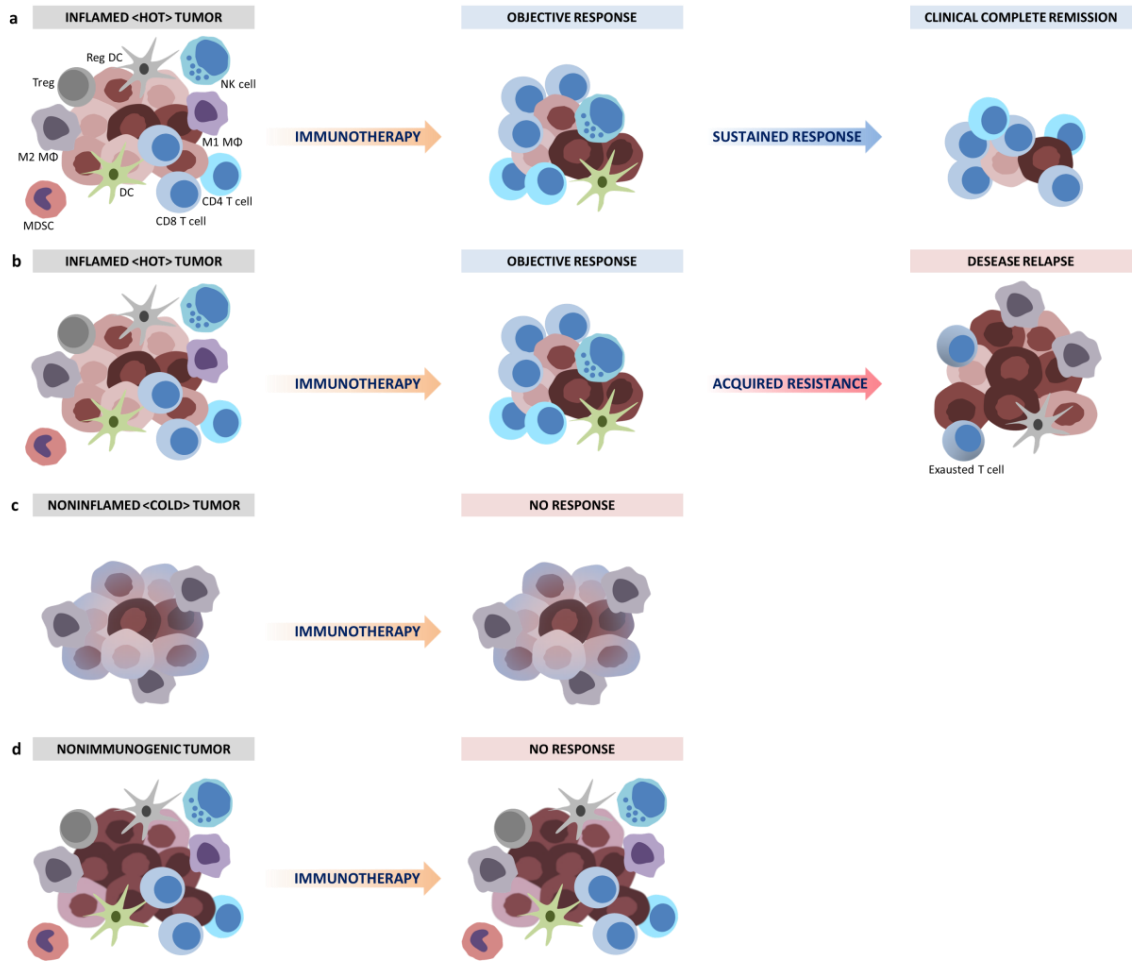

Figure 4. Cancer immunotherapy potential resistance scenarios. On **a,** in inflamed <hot>tumors, there's already a preexisting spontaneous infiltrate of CD8+ T cells and other immune cells that upon PD-1 checkpoint blockade immunotherapy have their effector function reestablished, leading to objective response and in the patients that enjoy durable sustained responses, clinical complete remission, in which the disease in no longer detected. Yet in contrast, on **b**, a significant fraction of patients, after achieving

objective responses, but not a sustained response, acquired resistance leads to diseases relapse. Alternatively, on **c** and **d**, characterizing two distinct scenarios of primary resistance in which the immune system is shown to be unresponsive to immunotherapy. On **b**, tumor intrinsic pathways, such as WNT/β catenin, were demonstrated to mediate T cell exclusion from the tumor, resulting in noninflamed <cold> tumor. On **d**, even in the presence of an immune infiltrate, immunotherapy still fails to provide objective response, which might be related to the wrong targeting of the immune system, alternative and unknown mechanisms of immune evasion or to tumors that harbor a low neoantigen load.

Building on complexity, other tumor intrinsic mechanisms associated to dampen the immune attack have also been observed, such as aberrant expression of PD-L1 (207), deficiency in the transporter for antigen presentation (TAP) protein (208), production of IL-1 via BRAF (V600E) mutation (209) and different alterations in type I IFN signaling (210-212), although subversion of the IFN pathway is gaining attention too. Indeed, persistent IFN-γ signaling was recently shown to regulate a multigenic resistance program mediated by an STAT-1 epigenetic signature that increases expression of multiple inhibitory ligands, including PD-L1 (213). Similarly, chronic expression of type I IFNs has been demonstrated to lead to the expression of IDO, PD-L1 and IL-10 (214).

On regard of extrinsic mechanisms that contribute to primary and acquired resistance, which we already have exemplified several ones throughout this review, in this section will focus on the role exerted by Treg cells. Identified by the expression of the transcriptional factor forkhead box P3 (FOXP3), $CD4^+$ Tregs also constitutively display CTLA-4 and the IL-2 high affinity receptor alpha chain (IL-2Rα, known as CD25) and have an essential function in preventing autoimmunity by maintaining immune tolerance to self-antigens (215). Accordingly, high infiltration of Tregs in tumors and, even more critically, a low ratio of CTLs to Tregs, is associated with poor clinical prognosis (216), as they employ an array of mechanisms to impair effector responses, rendering $CD8^+$ T cells tolerogenic for Ag stimulation, which is also a dysfunctional state (194, 215). Indeed, in addition to secretion of suppressive cytokines and IL-2 starvation, Tregs can exploit the CTLA-4 inhibitory pathway to impair $CD8^+$ T cell priming, either by outcompeting with binding of CD28 or by blocking $CD4^+$ licensing (217). Studies in mouse cancer models indicate that the antitumor activity of CTLA-4 blockade is partially mediated by FcγRIV-expressing macrophages that deplete tumor-infiltrating Tregs by ADCC (218, 219). Yet, in humans, evidence of CTLA-4 depletion of Tregs is not as clear.

Depletion of these suppressor cells is no easy task, owing to the lack of specific targets in tumor infiltrating Tregs, and potential risks of breaking peripheral tolerance. Cyclophosphamide is a well know chemotherapeutic agent that relies in its ability to

target Treg cells, when used in low-dose, but lacks specificity(220, 221). Aiming to circumvent this limitation, several agonist or depleting Ab based strategies have been developed aiming to modulate, if not eliminate, Tregs with more specificity (reviewed in (222)). Promising candidates being investigated in clinical trials include CCR4, which is predominantly expressed by effector, but not naïve, Treg cells, and is involved in Treg migration (223, 224); OX40 and GITR, which are constitutive inhibitory receptors expressed at high levels (225, 226); and as aforementioned, the CD25 receptor, for which an interesting improvement was recently reported by Vargas and colleges, where replacing the constant regions of the anti-CD25 Ab with murine IgG2a and κ constant regions (αCD2m2A) resulted in enhanced binding to FcγRs, effective depletion of intratumoral Tregs and synergism with PD-1 immunotherapy, eradicating established tumors (227).

Considering these mechanisms of primary resistance, it is safe to state that many obstacles must be overcome in order to modulate T cell immunity, and despite of them, long term responses have been observed by different immunotherapeutic strategies, especially remarkable was the durable effect obtained in response to Ipilimumab treatment in patients with advanced melanoma, that was shown to be maintained during ten years of follow-up (25). However, approximately 25% of patients treated with CTLA-4 or PD-1 blockade eventually develop resistance to therapy (228). Reasons for this phenomenon are largely unknown, but believed to rely on the ever evolving relationship between the immune system and cancer cells, as their various interactions are highly dynamic and plastic, with the potential to adapt to the pressure that each exerts on the other. Mechanistic evidence supporting this concept has been observed during melanoma progression as well as under immunotherapeutic pressure, indicating that inactivating mutations in the beta2-microglobulin (B2M) gene, an important structural protein of the human leukocyte antigen class I expression (HLA-I) complex, leads to lack of HLA-I expression and impairment of antigen recognition by $CD8^+$ T cells (229, 230). Along the same lines, IFNγ-resistant melanoma clones harboring inactivating mutations in *JAK1/JAK2* were shown to evolve into T-cell-resistant HLA class I-negative melanoma lesions with failed Ag presentation and insensibility to IFN-γ functions (231). Interestingly, JAK1 and JAK2 truncating mutations as well as a B2M frame shift mutation have recently been implicated in PD-1 resistance in relapsing melanoma tumors (232).

Failure to achieve sustained immunotherapeutic responses is also associated with up-regulation of alternative immune checkpoints, as recently demonstrated with TIM-

3(233), that, at least to some degree, cooperate with immunosuppressive molecules present within the TME to aggravate T cell dysfunction. In light of this concept and prompted by the success of combined CTLA-4 and PD-1 blockade, which has raised the bar for curative responses, a myriad of different combinatorial immunotherapy approaches is currently being intensively investigated in the clinical setting (a subject reviewed in greater detail in (234) and (235). Examples include combination with other inhibitory T cell checkpoints (e.g., anti-PD-1/PD-L1+anti-LAG-3), co-stimulatory checkpoints, that while applying immune brakes, are also pressing the "gas pedal' to promote T cell activity (e.g., anti-PD-1/PD-L1+anti-41BB, anti-CTLA-4+anti-OX40), metabolic modulators (e.g., anti-CTLA-4+IDO inhibitor, anti-PD-1/L-1+ adenosine receptor A2AR inhibitor), immune modulators (e.g., anti-PD-1/L-1+TGF-β inhibitor), NK activating mAbs (e.g., anti-PD-1/L-1+anti-KIR), DC based therapies (e.g., anti-PD-1/L-1+DC vaccine GVAX), targeted therapies (e.g., anti-CTLA-4+BRAF+MEK inhibitors), radiotherapy, chemotherapy (e.g., anti-CTLA-4+carboplatin), and epigenetic modifications (e.g., anti-PD-1/L-1+histone deacetylase inhibitors), among others (42).

Notably, as proposed by Melero and colleagues, the clinical efficacy of PD-1/PD-L1 immunotherapy and data from preclinicals models indicating its remarkable potential to be synergistically combined with other immunotherapies, if not all, the blockade of this pathway is likely to be the foundation of most combinatorial strategies (236). Interestingly, benefit for combined approaches may also be important for PD-1 blockade itself. As recently evidenced in previously untreated patients with stage IV or recurrent NSCLC positive for PD-L1 expression, PD-1 immunotherapy as first-line treatment, unexpectedly, was not associated with significantly longer progression-free survival in comparison to platinum-based chemotherapy (237). This is obviously a concerning result, that needs further elucidation, but also a tempting one, since it suggests that in previous clinical trials, pretreatment with chemotherapy may have influenced the response to PD-1 immunotherapy, which if clinically confirmed, may be particularly exciting, as chemotherapy is a standard of care treatment option. Most importantly, therapeutic failure in patients whose tumors were positive for PD-L1 expression underscores the urgent need to move the field to its era, where administration and combinations of immunotherapies are employed on a rational, mechanistic basis.

ENTERING INTO THE PERSONALIZED ERA

So far, we have discussed how to effectively induce the patient's immune system to fight cancer, but in order to achieve superior curative responses in a larger proportion of patients it will be fundamental knowing how to identify the most appropriate immunotherapy for each cancer patient and how to modulate immunogenicity in scenarios that are notorious for being refractory to immunotherapeutic approaches. Therefore, in this section we will *first* discuss prediction biomarkers. And, *secondly*, present evidence on how neoantigens can be targeted to augment immunity.

Cancer immunotherapy biomarkers

Before addressing the biomarkers themselves, consideration must be given to the interpretation of response to treatment and its relationship to the biomarker, as the predictive power of the biomarker may change due to transitional or fluctuating conditions during the immune response and measurement of biomarkers may best be performed at different time points during the treatment (238, 239).

A variety of cellular and molecular biomarkers have been examined as predictors of response to immunotherapy, including the location and characteristics of leukocytes, of which detection of TILs has become a reliable, positive prognostic indicator (240). The immunoscore is determined by evaluating $CD3^+CD8^+$ lymphocytes as well as memory T cells ($CD3^+CD45RO^+$) at both the tumor margin and at the core. The highest score, and best predictor of positive response to treatment, is associated with the presence of these cells at both the periphery and center of the tumor. The immunoscore was originally developed for colorectal cancer (CRC), as strong lymphocytic infiltration was associated to be a better predictor of patient survival than the histopathological methods currently used to stage CRC (241), but is being studied for broader application, an endeavor that will require improved processing of large numbers of samples and extensive amounts of data as well as an international consensus on interpreting and applying the findings to clinical choices (242, 243).

Pretreatment detection of PD-L1 expression in tumors was associated with a 48% response rate to blockade, supporting its use as a first-line therapy (244). Though 15% of PD-L1-negative tumors also responded, suggesting that PD-L1 detection cannot be used alone as a predictive biomarker (43, 244). Thus, determining PD-L1 expression can be predictive of response to anti-PD1/PD-L1, but requires a considerable degree of interpretation. First, the expression of PD-L1 is, in part, activated by IFN-γ produced by NK and activated T cells (245), suggesting that presence of PD-L1 in tumor cells may act as a defense raised due to a previous immune attack (43). PD1 pathway blockade,

therefore, may reinvigorate an existing population of experienced cytotoxic T cells. Response to anti-PD-LI was improved in patients with IFN-γ expression whereas PD-L1+IFN-γ positivity correlated with even better response (246). Defects in Jak1/2 correlate with tumor progression and lack of IFN-γ response(232). Alternatively, the expression of PD-L1 by tumor cells may be the result of DNA alterations, including oncogenic mutation and translocations (43, 240). PD-L2 may also be evaluated, but its expression is less frequent than PD-L1 in solid tumors, thus blockade of PD-1 would be a broader approach as compared to targeting PD-L1 or PD-L2 (43).

Examination of PD-L1 expressed on TILs has also been correlated with improved outcome in response to anti-PD-L1 immunotherapy. In the work of Herbst and colleagues (247), pretreatment PD-L1 expression on tumor infiltrating T cells, macrophages and DCs, but not B cells, was associated with better outcome. Increased PD-L1-positive TILs was more critical than positive tumor cells for predicting efficacy, yet PD-L1 was not upregulated in either cell population during treatment (247).

Looking beyond the checkpoints may also reveal predictive markers of response to immunotherapy. Though the evaluation of cancer antigens has long been studied, more recent approaches have uncovered not only markers, but targets for therapy. In the classic approaches, non-mutated gene products have been evaluated, including cancer germline antigens (expressed in embryonic and tumor tissues), differentiation antigens (for example, markers of melanocytes, MART-1 or pg100, found in melanoma), overexpressed proteins (such as carcinoembryonic antigen) (43, 240).

Newer approaches capitalize on whole-exome sequencing in order to reveal the full complement of transcripts in normal versus cancer cells, thus exposing somatic mutations. Indeed, higher mutation loads have been identified in cancers types associated with greater responses to checkpoint blacked immunotherapy, such melanoma, squamous lung cancer, NSLC and bladder (248). The key benefit of having elevated number of mutations is that it also brings an increase in tumor neoantigens, which are novel proteins sequences, harboring a neo-epitope, derived from tumor-specific mutations, and as so, are completely absent from the normal host genome and not subject to T cell tolerance like the classes of tumor associated antigens, which endows them to be perceived as foreign antigens, displaying high immunogenicity and T cell reactivity(249).

Pioneer evidence for the role that neoatingens have shaping tumor immunity was obtained from Matsushita and collaborators in 2012 by investigating trough exome and cDNA sequencing the expressed mutations in a highly immunogenic regressor

MCA derived sarcoma cell line, which among its 3,737 non-synonymous mutations they were able to identify a R913L point-mutation in the Spectrin-β2 gene as the main neoantigen that drives in a $CD8^+$ T cell dependent manner rejection of this cell line, its subclones that still harbors this mutation, but not progressor sub clones that had this neoantigen edited out and escape immunity control(250).

Soon after, in accordance to this observation large-scale analysis were carried out in order to determinate to the impact of neoantigens in response to immunotherapy, revealing that in melanoma patients neoantigen landscape is associated with a strong response to CTLA-4 blockade(251), correlates significantly with clinical benefit(252), and in NSLC patients, higher non-synonymous mutation burden is associated with improved objective response, durable clinical benefit, and progression-free survival of ant-PD-1 pembrolizumab(253). But, perhaps, the clearest support for impact of somatic mutations comes from mismatch-repair (MMR) deficient cancers that accumulate much higher numbers of mutations. As shown for CRC, MMR defects were associated with increased TIL, Th1 phenotype and expression of checkpoint proteins as well as IDO, predicting that PD-1 blockade would be effective (254, 255). Indeed, MMR deficiency in CRC was correlated with markedly improved response to pembrolizumab as compared to cases without MMR defects (256), and more recently, objective responses were observed in 53% of patients with MMR from 12 distinct tumor types, supporting the application of MMR as a universal biomarker for PD-1 immunotherapy, in disregard of the cancer tissue of origin (257).

Yet, the relationship between neoantigens and T cell response requires further investigation, as recently observed in melanoma tumor that density of immunogenic antigens does not explains the presence or absence of TILs(258), which can related to the mechanisms of T cell exclusion we already discussed. And as so, investigation of biomarkers will require that the techniques evolve to cost efficient, fast and user friendly approaches in order to encourage the wide-spread use of this technology. In addition, the sharing of data becomes critical in order to speed progress and shape future marker-driven trials (243).

Exploiting neoantigens for personalized cancer immunotherapy

On basis of the aforementioned evidence it is notorious that neoantigens have a core position in the cancer-immunity cycle. And considering technological advances that led for their identification it is now feasible to develop a pipeline for personalized cancer immunotherapeutic strategies **(Figure 5)**. Proof-of-principle for this appealing concept came from two seminal studies. The first, published by the Sahin group in

2012, aimed to identify the mutation landscape of the B16F10 mouse melanoma cell line and applied exome sequencing along with RNA-seq to identify 563 (out of 923) nonsynonymous somatic point mutations in expressed genes (259). Immunogenicity of these mutations was assessed by immunizing mice with synthetic long peptides (SLPs) encompassing the mutant epitope along with Poly I:C as adjuvant. Out of the 50 SLPs tested, 16 were shown to elicit immune responses specifically to the mutant sequence, but not the wild-type, indicating that a single nucleotide base substitution can strongly modulate the immunogenicity of a tumor antigen. Importantly, in a therapeutic vaccine model, SLP vaccination was able to provide antitumor protection, retarding tumor growth.

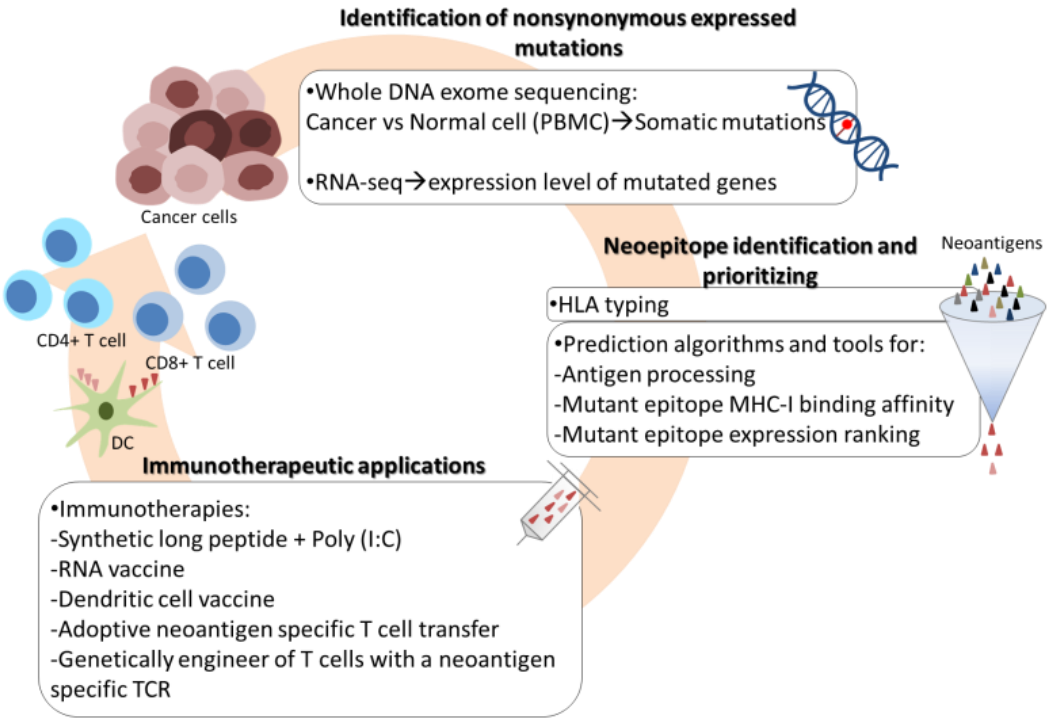

Figure 5. Identification pipeline for the development of neoantigen-based immunotherapy. After collection of cancer samples and peripheral blood mononuclear cells (PBMCs), tumor-specific non-synonymous expressed mutations are identified by whole DNA exome sequencing and RNA-sequencing, used to feed mutation calling algorithms. Next, after HLA typing, candidate neoepitopes are identified and prioritized according to computational tools for antigen processing, expression levels and their MHC-I biding affinities. These selected neoantigens can then be applied for different immunotherapeutic applications, including generating synthetic long peptides, RNA and dendritic cell vaccines. Alternatively, neoantigen-specific TCRs can also be isolated or generated to be adoptively transferred to the patient.

In the second publication, in 2014, Gubin and collaborators set out to identity the nature of tumor antigens that were targeted upon successful CTLA-4 and PD-1 immunotherapy in progressively growing MCA derived sarcomas (260). Exome and RNA-seq was used to identify 2,796 non-synonymous expressed mutations and, after MHC-I epitope prediction and filtering, two mutant epitopes with superior binding affinities were identified, a G1254V mutation in Laminin α subunit 4 (mLama4) and an

A506T mutation in Asparagine-linked glycosylation 8 (mAlg8). Dominance of mAlg8 and mLama4 antigen-specific $CD8^+$ T cells was revealed among the TILs by tetramer staining as well as superior secretion of IFN-γ and TNF-α. Notably, SLP therapeutic vaccination combining these two mutant epitopes with poly I:C resulted in regression of established tumors with a comparable efficacy as checkpoint blockade, thereby indicating that the predicted antigens were indeed capable of mediating antitumor responses and that neoantigen-based strategies can be used to develop personalized cancer-specific vaccines.

Supporting evidence was also reported in additional contemporary independent studies. Yadav and colleagues combined exome and RNA sequencing analysis with mass spectrometry to identify neo-epitopes in MC-38 and TRAMP-C1 mouse tumor cell lines, which were found to harbor 1,290 and 67 expressed non-synonymous mutations, respectively (261). Interestingly, out of theses mutations, just 7 (MC-38) and 0 (TRAMP-C1) were found to be presented by MHC-I examined by mass spectrometry. The contrasting numbers obtained by these two methods were thought to be related to the sensitivity of the mass spectrometry method, but this result also suggests a limit for the presentation of encoded peptides on MHC-I molecules. Indeed, 3 of 7 mass-spec predicted neoantigens elicited antigen-specific $CD8^+$ T cells and, by using SLPs and as adjuvant anti-CD40 Ab plus poly(I:C), vaccinations resulted in a striking anti-tumor protection in the prophylactic and therapeutic setting. Interestingly, through modeling of mutant peptide–MHC-I complexes, two of the three peptides used in the vaccination were shown to have their mutated residues oriented towards the solvent interface.

Kreiter and colleagues also made an interesting observation regarding neoantigen prediction (262). They analyzed three independent tumor cell lines, identifying neoantigen epitopes with high MHC-I binding, yet upon vaccination, these are in fact recognized principally by $CD4^+$ T cells (~80% of the epitomes evaluated). Moreover, seeking to demonstrate the feasibility of using mutant $CD4^+$ epitopes for therapeutic vaccination, they implemented prediction algorithms for MHC-II binding and engineered an RNA vaccine encoding one MHC-I restricted and four MHC-II tumor specific 27-mer amino acid epitopes (mutation was located at position 14), complexed this with cationic lipids before systemic application and, strikingly, showed efficient control over metastatic tumors.

In accordance with these observations, Schumaker and colleagues studied point mutations in the isocitrate dehydrogenase type 1 (*IDH1*) gene known to be associated with a subgroup of gliomas(263). The DH1(R132H) mutation was demonstrated to

harbor an MHC-II restricted epitope that elicited detectable $CD4^+$ T_h1 responses in patients harboring the IDH1(R132H) mutation, but not with its wild-type allele. And, in K.O mice lacking endogenous MHC, but engineered to express human HLA-A2.1-/HLA-DR1, SLP vaccination encompassing the mutant epitope resulted in growth suppression of established syngeneic IDH1 (R132H)-expressing tumors in a $CD4^+$ T-cell-dependent manner.

Taken together these studies highlight the potential of targeting tumor neoantigens in cancer immunotherapy. Yet, they also make evident the obstacles encountered for the prediction and validation of *bona fide* immunogenic neoantigens. For example, it was observed that immunogenic tumors express thousands of nonsynonymous mutations, but after functional validation, just a minority of those can actually be detected in the TIL pool and capable of inducing effective responses. And still on that note, in these preclinical vaccination experiments, targeting of just a few neoantigens seems to be sufficient for mediating tumor rejection, which argues for phenomena observed in viral diseases, such as antigen dominance and epitope spreading (reviewed in (264)), being involved in therapeutic responses in cancer as well.

Difficulties were also noticed in terms of prediction of CD4+ epitopes, as the majority the available algorithms are designed for MHC-I epitopes and the performance of MHC-II tools is considered less efficient and in more need of improvement (265). Part of the difficulty stems from the fact that MHC-II molecules can accommodate peptides longer than those presented by MHC-I, making prediction of alignment and identification of MHC-II motifs harder to determine(265). Moreover, prediction methods are mostly designed for point mutations and small insertions and/or deletions, but neoantigens can also arise from alterations in the reading frame, nonstop mutations, long DNA insertions and deletions, chromosomal translocations, phosphorylation and glycosylation of proteins (266). Consequences of these subsets of neoantigens on immunity are largely unknown.

In fact, recognition of a neoantigen is not just a reflection of high binding affinity, it also relies on how efficiently a given antigen is processed (immunoproteosomal cleavage and TAP transport), epitope position on the MHC-I molecule, and also, by the heterogeneity found in human alleles encoding MHC-I molecules, all of which are thought to have distinct peptide-binding capabilities (267). The integrative use of multiple algorithms along with different methods for antigen discovery, such as mass spectrometry, is expected to more accurately prioritize candidate neoantigens.

Within the bulk of heterogenous cancer cells that encompass the tumor, antigenicity may also be influenced by the concomitant expression of self-antigens, antigens derived from viruses (e.g., HPV oncoproteins E6 and E7), cancer-associated antigens and also by the neoantigen intratumor clonality, as recently demonstrated in a study by McGranahan and colleagues (268). They performed exome sequencing on multiple spatially separated primary lung adenocarcinoma tumors and the distribution of their neoantigens was analyzed, showing that the neoantigen landscape differs greatly across the tumor sections, as a substantial fraction was found homogenously expressed throughout the tumor (considered to be clonal); others were located in multiple tumor regions, but not all (shared subclonal), and the smallest fraction restricted to a specific region (private subclonal). Interestingly, high clonal distribution of neoantigens correlated with superior overall survival in response to PD-1 blockade and increased infiltration of antigen-specific $CD8^+$ T cells, suggesting that clonal and subclonal neoantigens have different roles in antitumor immune responses.

Nevertheless, these antigenic features may be exploited to mount effective neoantigen-specific immune responses in cancer patients. In the first report, the Rosenberg group subjected a lung metastasis from a cholangiocarcinoma patient to whole-exome sequencing and generation of minigenes encoding each of the 26 nonsynonymous mutations that were identified, which were later transfected in autologous APCs, co-cultured with TILs (obtained from the lung metastasis) and led to the detection of a dominant $CD4^+$ Th1 T cell response against an epitope found in the erbb2 interacting protein ($ERBB2IP^{E805G}$ mutation) (269). These T cells were then expanded, cloned and the patient infused with two rounds of neoantigen-specific ACT (25% specific in the first and 95% in the second), achieving tumor regression and stabilization of disease.

In another report, but now using a dendritic cell vaccine platform, three stage III melanoma patients had their tumors surgically resected and submitted to exome sequencing as well as cDNA capture analysis of predicted HLA-A*02:01 restricted antigens (270). Per patient, seven validated neoantigens were used to load an autologous, CD40-L/IFN-γ activated, 12p70-producing DC vaccine. Remarkably, it was noticed that vaccination increased the frequency of $CD8^+$ T cells specific for the targeted neoantigens and revealed responses to other neoantigens, with no previously detected immunity, thus indicating that vaccination strategies targeting neoantigens can induce a more broad repertoire of neoantigen-specific T cells, by modulating dominant and subdominants responses.

Accordingly, reports from two independent and concurrent phase I clinical trials present strong indications of therapeutic efficacy and safety. In one study by the Wu group, tumor neoantigens from patients with previously untreated melanoma were selected by HLA-I binding prediction, then 13–20 mutations per patient included in clinical grade SLPs and administrated in four pools along with POLY-ICLC adjuvant, 18 weeks after surgical resection (271). Adverse effects were considered as mild, mostly involving flu-like symptoms, rash and fatigue. Of the six vaccinated patients, four present no recurrence of disease and two eventually relapsed, but upon PD-1 blockade both achieved sustained responses accompanied with a broader neoantigen repertoire of T cells. Interestingly, after vaccination, the secretion of IFN-γ, TNF-α and IL-2 by poly-functional $CD4^+$ and $CD8^+$ T cells was observed specifically in response to the neoantigens, which responses against the latter were undetectable in samples collected before vaccination and, in their majority, were driven by MHC-II epitopes (60%) than MHC-I (20%). Indeed, comparison of transcriptional profiles of antigen-specific CD4+ T cells pre and post vaccination indicated major shifts in programs of effector and memory functions.

In the second report, by Sahin and collaborator, each of the thirteen melanoma patients received up to 20 vaccine doses containing ten selected neoepitopes, which were engineered into two pentatope synthetic RNAs (272). Notably, treatment was well tolerated, and 60% of the predicted neoepitopes were shown to induce responses, being two thirds of them through a *de novo* mechanism and mainly mediated by $CD4^+$ T cells, with a smaller fraction restricted to $CD8^+$ and, interestingly, also to concomitant $CD4^+$ and $CD8^+$ responses, recognizing different epitopes from the same peptide. Importantly, antigen-specific $CD8^+$ T cells were indicated as having a CTL phenotype, with high proliferation and effector-memory functions. During the follow up, all patients benefited from reduced cumulative sum of recurrent metastatic events and prolonged progression-free survival. Among the cases, eight remained without recurrence and two enjoyed complete response. Another one initially responded but eventually developed acquired resistance by the loss of both B2M alleles. A third patient, who was experiencing fast disease relapse post-vaccination, evolved to complete response after combination with anti-PD-1 immunotherapy. Therefore, just as in the aforementioned study, suggesting that further development of personalized cancer vaccines based on neoantigens is warranted their combination with checkpoint blockade or possibly with other immunotherapies.

CONNECTING IMMUNITY TROUGH ITS TARGETS

Given what has been discussed to this point, there are several promising targets within the cancer-immunity cycle and the combination of these targets will be a crucial step forward for the field.

Careful design of the treatment regime is necessary in order to achieve optimal activity as well as to limit immune-related adverse events (irAE), which include elevated levels of inflammatory cytokines in the circulation, gastrointestinal, dermatological and endocrine toxicities (41). To this end, studies of dose optimization (i.e., different as compared to use as monotherapy), schedule (i.e., sequential, concomitant, order) and the combination components, are certainly important, especially taking into consideration the different toxicity rates observed across the spectrum of cancer types (273). For example, in NSCLC patients treated with the same dose and schedule of nivolumab plus iplimumab as compared to melanoma and RCC patients, 49% were shown to present severe toxicity and 35% discontinued the treatment, which eventually required the use of lower doses and longer intervals of treatment (234, 274). Additionally, adverse events also seem to be related specifically to the agent used, as different irAEs were noted for PD-1 and CTLA-4 blockade, being hypophysitis the most frequent endocrine irAE for CTLA-4 and hypothyroidism for PD-1 blockade (41). Yet, the mechanisms behind these adverse effects are not completely understood, but thought to depend on checkpoint blockade for breaking immune tolerance to self-antigens and tumor antigens. A notion that is supported by the observation of on dyspigmentation upon CTLA-4 blockade in melanoma bearing mice that, due to epitope spreading mechanisms, elicit a CTL attack against self-melanocyte antigens expressed by the tumor cells and thus drive autoimmunity to normal melanocytes (21). Activation of pathways like CCR5/CCL-3-5, CXCR3/CXCL-9-11 and STAT-1 IRF1, observed during the immune attack of tissues in autoimmune conditions, such as rheumatoid arthritis, allograft rejection and GVHD, can also be detected during the antitumor immune responses (275). This intriguing similarity may point to an autoimmune limit to the number of checkpoints can be targeted in combination and also suggests that association with other treatment modalities may be crucial for providing safer yet equally effective results, especially in the case of neoantigen-based vaccines, which target antigens not expressed in normal tissues.

Although there is no consent on which immunotherapy will be best for combination with neoantigen vaccines, the use of CTLA-4 blockade is an appealing option since it has been shown to broaden the peripheral T cell receptor repertoire (276)

and increase the number of detectable melanoma-specific $CD8^+$ T cell responses (277). Therefore, responses to sub-dominant neoantigens may accumulate while avoiding the development of acquired resistance due to the selective loss of neoantigens, as has been recently reported upon ACT in melanoma patients (278). Notably, two patients in this study achieved immune escape, and consequent disease relapse, one due to the loss of heterozygosity at the locus harboring the neoantigen allele RPL28S>F and, in the second patient, due to a dramatic reduction in expression of the gene encoding the SEPT2R>C neoantigen, reflected in decreased accumulation of $CD8^+$ T cells specific for these neoantigens in the TIL, but interestingly there was an increase in other tumor reactive T cells.

Therapeutic inefficacy of ACT in melanoma patients has also been reported to involve coordinated functional dynamics between adoptively transferred T cells and endogenous T cells that undergo a progressive loss of their poly-function ability to secrete granzyme B, IFN-γ, and TNF-α (279). Trafficking of effector T cells into tumors was recently associated with the production of CXCL9/10 by $CD103^+$ DCs upon activation of the STING-type I IFN pathway and, conversely, lack of accumulation of $CD103^+$ DCs within the tumor was indicated as a mediator of ACT immunotherapy resistance (280, 281). Both observations highlight the importance of promoting an immune stimulatory TME that favors infiltration and maintenance of effector cells functionality, which in "inflamed" tumors has been shown to be achieved by doublets of CTLA-4 of PD-L1 blockade (143), CTLA-4 and IDO blockade (282) or by the production of IFN-β induced by local radiotherapy in combination with PD-1 immunotherapy (283).

Preclinical studies have revealed models in which formation of a highly immune suppressive TME turns established tumors refractory to the combination of just two immunotherapies, more closely resembling the advanced stage at which human tumors are clinically detected. For example, immune rejection of establisedTC-1 tumors, a model of cervical carcinoma induced by HPV, required the use of a tripletherapy composed of CpG complexed with a cationic lipid (TLR9 agonist), low-dose cyclophosphamide (depletion of Tregs) and vaccination against the E7 viral antigen delivered by adenylate cyclase (CyaA) (284). In another example, radiation therapy given together with CTLA-4 and PD-1 checkpoint blockade was shown to have distinct effects on the TCR repertoire, Treg cells, T-cell exhaustion and consequently, to mediate complete responses in different cancer models (285). More recently, tripletherapy including oncolytic herpes simplex virus (HSV) expressing IL-12

combined with CTLA-4 and PD-1 dual blockade was demonstrated to effectively eliminate gliomas by promoting the contribution of M1 macrophages to the attack mediated by $CD4^+$ and $CD8^+$ T cells (286).

However, combinations such as these may not be effective for "cold tumors" or for tumors that display a low mutation burden, which as we already discussed fail to respond to immunotherapy. Both of these features can be observed in specific genetic models of cancer, where activation of oncogenic programs leads to exclusion of T cell infiltration or low neoantigen load. Indeed, in the KrasLSL-G12D/+;Trp53flox/flox conditional genetic model for NSCLC (driven by Kras and Trp53 mutations), combination of oxaliplatin chemotherapy (inducer of ICD) with cyclophosphamide (depletes Tregs) could render orthotopic tumors, lacking pre-infiltrated T cells, sensitive to CTLA-4 and PD-1 dual blockade through a mechanism dependent on TLR4 innate immune sensing and on $CD8^+$ T cells (287). Along these same lines, Moynihan and colleagues explored the *BrafCA PtenloxPTyr::CreERT2* inducible melanoma model (induced by BrafV600E and Pten mutations) to demonstrate that tumor eradication required the complementary properties of four distinct immunotherapies, comprised of an Ab against the melanoma-associated antigen tyrosinase-related protein 1 (Trp1) to mediate ADCC, an extended-half-life recombinant IL-2 (expands and enhance functions of T and NK cells), anti-PD-1 immunotherapy as well as a trivalent amphiphile–vaccine targeting gp100, Trp1 and Trp2 tumor-associated antigens (288). Depletion experiments revealed that antitumor immunity was dependent on the cooperation of innate and adaptive components, $CD8^+$ T cells, NK cells, macrophages, but not on $CD\text{-}4^+$ T cells. Interestingly, it was also noticed that cross-presentation by $CD103^+$ DCs and epitope spreading augmented the breadth of antigens encoded by the vaccine and promoted *de novo* responses.

It will be interesting to evaluate how neoantigen-based immunotherapies could be exploited in tumors such as these that exhibit a low mutation burden. Take for example Merkel cell carcinoma (MCC), which can be caused by UV-induced somatic mutations or by the Merkel cell polyomavirus (MCPyV). Even though the virus-induced carcinoma presents a low mutation burden that would indicate its inability to respond to checkpoint blockade immunotherapy, it actually shows a high objective response rate of 56% to PD-1 blockade that is comparable to $MCPyV^{negative}$ MCCs(289), suggesting that neoantigens derived from viruses can be effectively targeted. Thus, from a conceptual point of view, the argument may be made that if a neoantigen is immunogenic enough and at the same time vital for the maintenance of the malignant phenotype, as seen with

MCPyV, a high neoantigen burden may not be necessary for driving robust responses. Another example comes from a murine model of pancreatic cancer where low immunogenicity resulting from the lack of neoepitope burden could be reversed by *ex vivo* introduction of a strong non self-antigen, establishing checkpoint sensibility(290). If response indeed correlates with "quality" and not 'quantity', neoantigens derived from driver mutations could be identified, providing a shared class of neoantigens with a more "universal" applicability instead of relying on the accumulation of random, passenger neoantigens.

Building in complexity, a recent study by Spintzer and colleagues indicated that generation of memory T cells in the periphery is required for sustaining effective responses (291). In their study, PD-L1 blockade resistant, MMTV-PyMT murine mammary tumors were treated intratumorally with tumor-binding Abs combined with anti-CD40 and IFN-γ DC adjuvants and organism-wide analyses were carried out, showing that the TME and peripheral secondary lymphoid organs undergo distinct immune dynamic events during priming and the effector phases of the immunotherapy. Interestingly, despite initial high expansion of macrophages, NKs and T cells in the TME, it was observed that throughout the tumor rejection phase, cell proliferation in the draining LN was mostly associated with effector memory Th1 $CD4^+$ T cells ($CD44^+CD69^+CD62L^-CD27^{low}T\text{-}bet^+$), which were rapidly expanding and provided antitumor protection upon transfer to new tumor bearing host, thus indicating their dominant role in orchestrating systemic immunity.

In simplistic terms, the main role of memory T cells is thought to be in the combat of metastasis and relapse, but it is clear that much remains to be understood about their exact functions, as studies are indicating the importance of other memory T cells subsets, including the interplay between resident and circulating memory $CD8^+$ T cells (292), central memory $CD8^+$ T cells (293), and $CD8^+$ effector memory T cells, shown to be expanded in melanoma patients that respond to PD-1 blockade (294). Moreover, mounting evidence also indicates the involvement of the gut microbiota in dictating the efficacy of chemotherapy (295) and checkpoint blockade (296, 297), arguing again for a systemic immunity modulation. There is also an increasing interest in the conditions leading to the formation of tertiary lymphoid organs (TLO) within the tumor mass, since TLO have been observed to provide a functional environment for effective T cell recruitment and priming (298), thus, at least on theoretical grounds, circumventing the need for T cell homing and infiltration.

CONCLUDING REMARKS

Taken together, the studies discussed here reveal remarkable achievements in unveiling the nature of tumor immunity. Now that our understanding of the interactions between cancer cells and immune system has grown to an astonishing complexity, we are able to target immunity at its most fundamental levels. The capability to detect non-synonymous expressed mutations, predict presentation and antigenicity of the corresponding peptides and then develop a personalized cancer vaccine represents an impressive advancement.

In fact, the cancer immunotherapy tool box has never before held so many promising options. Yet, in sharp to contrast to our treatment strategies, the myriad mechanisms of immune evasion, being primary or acquired, have also never seemed so challenging. And taking into consideration the *e*volutionary pressure exerted by therapeutic immune responses and the inherent cancer heterogeneity and plasticity, it is safe to say that much more remains to be discovered in this matter.

It is also becoming clear that to achieve the full potential of cancer immunotherapy in a broader number of patients and tumors types, the use of immune checkpoint blockade, even in combination, will not be enough. Eliciting T cell responses will also fall short unless adaptive resistance mechanisms can be circumvented, thus sustaining functionality within the tumor mass and in the periphery. Towards this end, advances in biomarkers and methods for immune monitoring are expected to take the field into the era of rationalized treatment. Optimal combinatorial approaches are also poised to significantly increase therapeutic efficacy. We hypothesize that these combinations will likely be tailored according to the requirements of each patient and targeting of innate and adaptive immunity will be essential to orchestrate long lasting systemic responses **(Figure 6)**. Despite the large number of discoveries yet to be made, or perhaps because of them, the coming era will certainly be a very stimulating one, with steady progress in the field of immunotherapy.

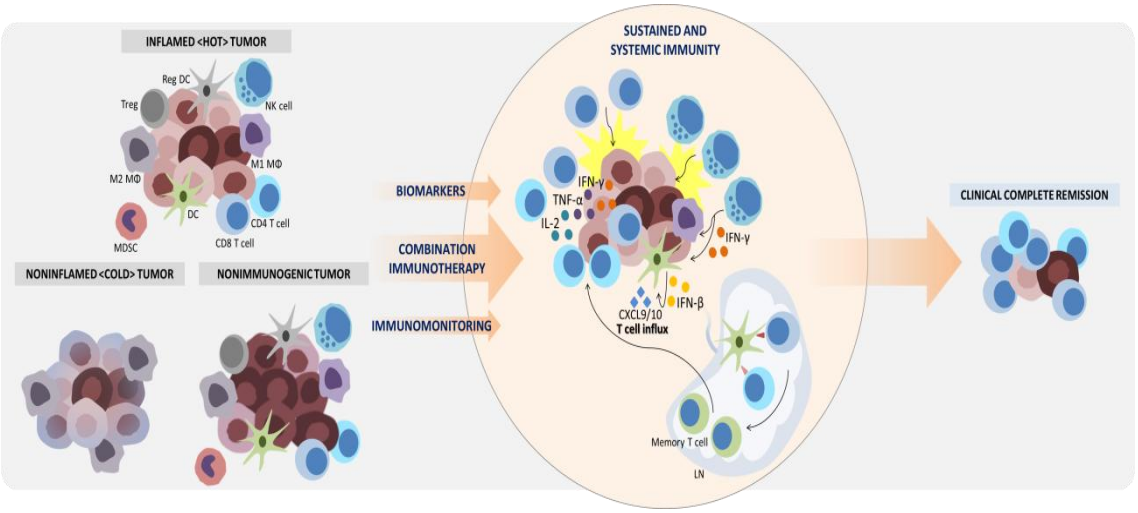

Figure 6. Eliciting the full potential of cancer immunotherapy. Currently, just a fraction of patients and cancer types respond to cancer immunotherapy and in order broad this number, we propose that the associated use of biomarkers, methods for immune monitoring with optimized combinatorial approaches can elicit effective T cell immunity in unresponsive scenarios. And towards this end, sustaining T cell influx and effector function within the tumor microenvironment as well as in the periphery can be considered as a critical step for achieving systemic immunity and significantly increasing therapeutic efficacy.

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Immunomodulatory and antitumor effects of type I interferons and their application in cancer therapy

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Review

Immunomodulatory and antitumor effects of type I interferons and their application in cancer therapy

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ABSTRACT

During the last decades, the pleiotropic antitumor functions exerted by type I interferons (IFNs) have become universally acknowledged, especially their role in mediating interactions between the tumor and the immune system. Indeed, type I IFNs are now appreciated as a critical component of dendritic cell (DC) driven T cell responses to cancer. Here we focus on IFN-α and IFN-β, and their antitumor effects, impact on immune responses and their use as therapeutic agents. IFN-α/β share many properties, including activation of the JAK-STAT signaling pathway and induction of a variety of cellular phenotypes. For example, type I IFNs drive not only the high maturation status of DCs, but also have a direct impact in cytotoxic T lymphocytes, NK cell activation, induction of tumor cell death and inhibition of angiogenesis. A variety of stimuli, including some standard cancer treatments, promote the expression of endogenous IFN-α/β, which then participates as a fundamental component of immunogenic cell death. Systemic treatment with recombinant protein has been used for the treatment of melanoma. The induction of endogenous IFN-α/β has been tested, including stimulation through pattern recognition receptors. Gene therapies involving IFN-α/β have also been described. Thus, harnessing type I IFNs as an effective tool for cancer therapy continues to be studied.

INTRODUCTION

Type I interferons (IFNs) are pleiotropic immunomodulatory cytokines that were originally described based on their ability to interfere in the viral infection cycle [1], that is to say, activate protective antiviral machinery in infected cells, their neighbors and, on a systemic scale, in antigen presenting cells (APCs) and T lymphocytes [2]. The IFN family is subdivided into three types of cytokines — type I, type II and type III — which differ in their protein sequence, function, producer cell and cognate receptor. In humans, type I IFNs contain 18 distinct members (13 subtypes of IFN-α and one for each IFN-β, IFN-ε, IFN-κ, IFN-τ and IFN-ω) that, interestingly, all bind to the same cognate receptor, composed of the IFN-α/β receptor 1 (IFNAR1) and IFNAR2 subunits [3, 4]. Unlike the other type I IFNs, IFN-α and IFN-β have much more established and known roles in immunity and our review will focus on them. There is only one type II IFN — identified as IFN-γ — which binds to the IFN-γ receptor 1 (IFNGR1) and IFNGR2 subunits and is mainly produced by $CD4^+$ helper T lymphocytes and natural killer (NK) cells. Type III IFNs consist of IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4, which bind to the IFN-λ heterodimeric receptor 1 (IFNLR1) and Interleukin-10 (IL-10) receptor subunit β [3, 4].

During the past decades, a growing body of evidence clearly indicates that type I IFNs also play a pivotal role in naturally occurring and therapy induced immune responses to cancer [5]. This conclusion is based on two key observations:

First, *Ifnar1* knockout (KO) mice are more tumor-prone upon exposure to the carcinogen methylcholanthrene (MCA) in comparison with mice that have functional type I IFN signaling. Second, tumors that arise from this IFN-α/β deficient context were more immunogenic (i.e., immune rejected when transplanted into a immunocompetent, naïve, syngeneic host) than when they were originated in the wild type background, thus demonstrating a significant role for type I IFNs in immune surveillance during carcinogenesis and tumor progression [6].

The ability of the immune system to eliminate nascent transformed cells, control and sculpt the immunogenicity of developing tumors while in a state of equilibrium, and upon escape of the immune control, exert pro-tumor functions, are all contemplated in the cancer immunoediting hypothesis [7, 8]. And among the cellular and molecular pathways identified so far, type I IFNs seem to be critical components for the host immune response against tumor, more specifically for the dendritic cell (DC) compartment [6, 9].

First identified by Steinman and Cohn [10, 11] DCs are professional APCs that act as central regulators of the antitumor immune cycle [12]. While in the steady state, DCs are present in their immature form, characterized by high capacity to capture antigens, but low secretion of cytokines and expression of co-stimulatory molecules (e.g., CD80, CD40, CD86). Yet, in the face of tissue injury, cell death or microbial infection, DCs are activated and migrate to the draining lymph nodes (LN) where they acquire fully mature phenotype (i.e, high expression of major histocompatibility complex (MHC) molecules and co-stimulatory signaling potential). DCs will then pass on the message received in the microenvironment where the antigen was encountered, delivering both antigenic (through MHC-I and MHC-II, due to their cross-presentation ability) and co-stimulatory signals, via membrane and secreted molecules, such as CD80, CD86 and IL-12, respectively, to prime naïve T cells [13, 14]. Interestingly, spontaneous immune responses to tumor cells have been shown to depend on the activation of DCs by type I IFNs [15] and as a result, one of the first cancer immunotherapies ever to be approved by the US Food and Drug Administration (FDA) consisted of high doses of recombinant IFN-α2b for melanoma and renal cell carcinoma [16]. Since then, numerous other antitumor strategies have exploited the immunomodulatory properties of type I IFNs to bring the full force of the immune system to the cancer fighting arena. For these reasons, in this review we will discuss the pleiotropic effects of type I IFNs on cancer and immunity and some of the therapeutic opportunities based on this critical interaction.

DENDRITIC CELL SUBSETS IN CANCER

All DCs originate from bone marrow hematopoietic stem cells through sequential steps of differentiation that first form a common progenitor of macrophages/DCs and, secondly, give rise to two lineage specific precursors, one for monocytes and the other for DCs. The latter finally branches out into two major subsets, plasmacytoid DCs (pDCs) and conventional DCs (cDCs), which are further divided into cDC1 and cDC2, based on the transcription factors that drive the development process, cell surface markers and functions [17]. It is important to stress that much of the following nomenclature was obtained from studies of the mouse immune system and not all data from murine models perfectly match with their human counterpart.

In the mouse, cDC1s are negative for the CD11b marker, dependent on the inhibitor of DNA binding 2 (ID2), interferon regulatory factor 8 (IRF8) or basic leucine zipper ATF-like transcription factor 3 (BATF3) transcriptional factors, express the X-C Motif chemokine receptor 1 (XCR1) and display a remarkable capacity to cross-present antigens on MHC-I to activate $CD8^+$ T cell responses. Among the BAFT3 driven DCs, $CD8\alpha^+$ DCs are localized in lymphoid organs, such as spleen and LN, thus not found in the non-lymphoid organs, whereas $CD103^+$ cDCs are found in non-lymphoid organs [18, 19]. Importantly, *Bafi3* KO mice, which lack both $CD103^+$ and $CD8\alpha^+$ cDCs, when transplanted with highly immunogenic tumors (i.e., spontaneously regresses after being inoculated in immunocompetent mice) are not able to reject them [20] and even more critically, do not respond to checkpoint blockade immunotherapy [21]. On the other hand, cDC2s that can induce innate lymphoid cells (ILCs) and a Th2 immune response against multicellular parasites are a $CD11b^+$ heterogeneous population, dependent on the IRF4 and zinc finger E-box binding homeobox 2 (ZEB2) transcription factors, express the signal regulatory protein alpha (SIRPα/CD172a) transmembrane protein and present MHC-II antigens to $CD4^+$ T cells [15, 18]. Yet, their role in cancer remains unclear [15, 18, 19].

In humans, equivalent BAFT3 dependent DCs are identified by the expression of CD11c, CLEC9A, XCR1 and CD141 [22], have been found in different tumor types and, just as the murine DC, seem to be relevant in anti-tumor responses, since their presence correlates with a superior outcome in melanoma patients [14, 23]. Key insights on why these $CD103^+$/$CD141^+$ DCs display such unique function came from a recent work that used a mouse model of cancer to question which of the different tumor associated APCs (resident $CD11b^+$, migratory $CD11b^+$, $CD8\alpha^+$, $CD103^+$ and macrophages) could phagocytose ovalbumin (OVA) and m-cherry from tumor cells, migrate to the draining LN and still be positive for m-cherry fluorescence, indicating the presence of the intact antigen. Remarkably, only in the $CD103^+$ DCs subset could m-cherry fluorescence be detected. Upon isolation of APCs from the draining LN, once again, just $CD103^+$ DCs were able to drive T cell responses against the OVA antigen. Furthermore, it was shown that C-C

motif chemokine receptor 7 (CCR7) is required for CD 103^+ DCs to traffic tumor antigens to the LN and that CCR7 levels correlate with T cell infiltrate and patient survival [24].

The other important subset is comprised of pDCs, whose development is driven by the E2-2 transcription factor and, curiously, morphologically resemble plasma cells. The pDCs specialize in producing and secreting large amounts of IFN-α after pathogen stimulation of toll-like receptor 7 (TLR7, detects single stranded viral RNA) and TLR9 (double stranded DNA), thus having a relevant role in the innate immune response against viruses [15, 18, 19]. In mice, pDCs are mostly found in blood and spleen, categorized by the expression of B220, Ly6C and the plasmacytoid dendritic cell antigen-1 (PDCA-1) markers and in humans, while negative for T, B and NK cell markers, pDCs are positive for CD4, CD123 (IL-3R), CD303 (BDCA-2), immunoglobulin-like transcript 3 (ILT3), and ILT7] [25]. Though the available data do not show a clear role for them in antigen presentation and initiation of the adaptive immune response, they are able to present antigens in the context of MHC-II molecules [15, 18, 19]. Indeed, upon activation by viruses, cytokines such as IL-3, CD40L or CpG oligonucleotide pDCs differentiate into full DC morphology and activate $CD8^+$ and $CD4^+$ T cell responses [26, 27]. Interestingly, in different types of tumors, including melanoma and prostate cancer, pDCs have been shown to be present in reduced frequency in the circulation, as they express multiple chemokine receptors, such as CXCR4 and ChemR23, that determine their tropism for sites undergoing pathological processes [28, 29], a subject thoroughly reviewed in [27] and [28].

However, in spite of suggestions that they may be involved in the initiation of the response by producing IFN-α in the tumor microenvironment, their role is still not convincing and, actually, some human studies have even associated pDC infiltration of tumors with poor survival [14, 30]. Accordingly, for reasons not fully understood, tumor-associated pDCs display a reduced responsiveness to TLR9 stimulation, become defective in IFN-α production and secrete immunosuppressive factors (e.g., IL-10) that along with regulatory T cells participate in immune surveillance escape, hence, favor tumor progression [27, 31]. Along the same lines, Le Mercier and colleagues used an orthotopic murine mammary tumor model to show that depletion of pDCs retarded tumor growth, evidencing their pro-tumor role. Remarkably, instead of TLR9, intratumoral administration of TLR7 ligand activated tumor associated pDCs and provoked a strong tumor regression effect [32]. Depletion of pDC and neutralization of type I IFNs prevented this outcome. Nevertheless, production of IFN-α by human pDCs can also be negatively regulated through the receptors BDCA2, NKp44 and ILT7, although only ILT7 has a known ligand, BST2 found on the cell membrane upon exposure to type I IFNs and also in a fraction of melanomas [27, 33].

Monocyte derived DCs (moDCs), or inflammatory DCs, originate from circulating monocytes that are thought to be drawn to the inflammatory cancer microenvironment since they are not present in a steady state [34]. moDCs express the Ly6C marker in mice or $CD14^{high}$ in humans, but since in the mouse they are also positive for MHC-II, CD11b, CD11c and F4/80, it is hard to discriminate them from other $CD11b^+$ populations or macrophages. Currently, CD64 (high-affinity IgG receptor gamma chain FcγRI) and MAR-1 (high-affinity IgE receptor FcεRIα chain) staining is used to discriminate moDCs from $CD11b^+$ cDCs [14, 15, 34]. The capacity of moDCs to activate näive T cells requires further elucidation, since depending of the context and cytokines that are present, $Ly6C^+$ monocytes can give rise to both macrophages and DCs [15]. Additionally, in tumors, $Ly6C^{high}$ monocytes can remain as a heterogeneous population called myeloid-derived suppressor cells (MDSC) and through up-regulation of nitric-oxide, arginase, prostaglandin-E2 and production of transforming growth factor β2 (TGF-β2) can deeply impair function of effector immune cells [14, 35].

Regarding the infiltration of other DC subtypes into the tumor mass, data indicates that cDCs represent a scarce population, and as such are likely competing with other more abundant myeloid populations, such as macrophages and monocytes, for antigen capture and priming of T cells [36, 37]. In mouse models of cancer, the localization of most DC subtypes have been shown to be mostly localized in the tumor margins, with limited infiltration into the center [38]. In humans, owning to the difficulty to characterize the DCs *in situ*, their scarcity as well as cancer related heterogeneity, the localization of DCs remains poorly studied. Even so, in melanoma, peritumoral DCs have been observed which a more mature phenotype than infiltrating DCs' [39, 40]. However, in a recent breakthrough, Lavin and collaborators aiming to determine the immune landscape of early lung adenocarcinoma lesions, used a multiscale immune profiling strategy based on mass cytometry by time of-flight (CyTOF) combined with single-cell transcriptomics and multiplex tissue imaging and observed that $CD141^+$ DC (categorized by the high levels of CD207, CLEC9A, and XCR1) are significantly depleted in comparison with non-lung adenocarcinoma tissue, whereas $CD1c^+$ DCs (expressing CD1c, CX3CR1 and IRF4) were observed more frequently [41]. However, the impact of tumor infiltrating DCs on clinical outcome needs further investigation, as other cells present in the tumor stroma are also playing a role, a topic that was thoroughly discussed in [40].

TYPE I INTERFERONS SIGNALING PATHWAYS

In humans, the type I IFN family includes proteins encoded by at least 13 IFN-α genes (IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17 and -α21)

and one gene each for IFN-β, IFN-ε, IFN-κ, IFN-ω and IFN-τ [42]. Interestingly, IFN-α genes share 70–80% sequence homology and have about 35% identity with the IFN-β gene [43]. All type I IFN genes lack introns and are located on the short arm of chromosome 9 in humans and chromosome 4 in mice. IFNs-α and IFN-β have 186–190 amino acids, but they have a peptide that signals cleavage resulting in secreted proteins of 165 or 166 amino acids with the amino terminal domain being important for biological activity [44].

The various type I IFNs have differential tissue expression and although they bind to the same receptor (IFNAR1/IFNAR2) and signal through similar mechanisms [4, 45], they have different binding affinities and, consequently, give rise to different antiviral, antiproliferative, and immunomodulatory outcomes [46–48]. IFN-β has a ~50-fold higher receptor-binding affinity to IFNAR1 than IFN-α [49], resulting in a more potent antiproliferative and perhaps distinct immunoregulatory action [47]. Interestingly, only IFN-β, but not IFN-α, stimulation enables the co-immunoprecipitation of IFNAR1 and IFNAR2 subunits [50]. Also, the IFNAR2 subunit binds type I IFNs with relatively higher affinity than IFNAR1, but the latter is absolutely required for signal transduction [42, 51, 52].

The IFNAR1/IFNAR2 receptor consists of transmembrane proteins which lack intrinsic kinase domains. They associate with a family of nonreceptor cytoplasmic tyrosine kinases, the Janus kinases (JAK1 and TYK2), so they can phosphorylate specific tyrosine residues of signal transducer and activator of transcription (STAT) proteins [53]. TYK2 associates with IFNAR1 while JAK1 acts in association with IFNAR2 [52, 54] on the inner side of the membrane, thus providing stability to the receptors and facilitating their cell surface localization, while serving as key components of signaling complexes [55, 56]. These complexes phosphorylate Tyr701 in STAT1α and in its spliced variant STAT1β, and Tyr690 in STAT2, enabling p-STAT to form heterodimers via their Src homology 2 (SH2) domains (Figure 1) [57, 58].

Once phosphorylated, the heterodimer STAT1/STAT2 binds to IRF9, forming the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex [59]. The ISGF3 complex translocates to the nucleus and binds to *cis*-acting IFN-stimulated response elements (ISREs) in the promoters of IFN-stimulated genes (ISGs). While STAT1 and STAT2 require phosphorylation to be active, IRF9 functions as a DNA adapter molecule independently of its posttranslational modification induced by IFNα/β [60, 61].

Expression of type I IFNs is intimately connected and influenced by IRFs, a family of nine transcription factors with a similar DNA binding domain in their N-termini. IRF1 is expressed constitutively and also in response to IFN-γ, like IRF8, and it may determine which species of IFNs are induced by TLR activation. IFN-β induces synthesis of IRF7, which amplifies the synthesis of IFNs together with the constitutive expressed IRF3 [62, 63], and induces transcription of IFN-α genes [64].

In the type II IFN-γ signaling pathway, the homodimer of tyrosine-phosphorylated STAT1 binds to Gamma-Activated Sites (GAS) in ISG promoters [65]. Type I IFNs can also induce this pathway, triggering expression of genes with GAS in their promoters. Also, STAT1 can form heterodimers with other STATs leading to activation of other pathways (Figure 1) [66].

Differences between IFN-α and IFN-β and their antitumor effects

Type I IFNs can induce expression of different genes depending on their concentration. Some genes are highly sensitive and require low picomolar concentrations, while other genes require 100-fold higher IFN-α/β concentrations for activation. Microarray analysis of expressed genes revealed that antiviral activity genes (such as Mx dynamin like GTPase 1 - *Mx1*, protein kinase R - *PKR* and 2′-5′-oligoadenylate synthetase 2 - *OAS2*) are induced by low amounts of IFN-α/β, whereas genes related to cell proliferation, chemokine activity and inflammation (like *IL-6*, C-X-C motif chemokine ligand 11 - *CXCL11*, and tumor necrosis factor related apoptosis inducing ligand - *TRAIL*) need a stronger IFN-α/β signal in order to be activated [47, 48, 67, 68]. The difference between antiviral and antiproliferative activities for IFN-α2 was 1,000-fold, while for IFN-β it was only 50-fold in WISH cells [51].

IFN-β binds to IFNAR1/IFNAR2 with higher affinity and thus forms more stable ternary complexes than IFN-α [47, 48, 69]. Because of this, IFN-β regulates cellular functions at concentrations that are orders of magnitude lower than any IFN-α subtype. Yet, all type I IFNs can induce antiviral responses at picomolar concentrations [69, 70].

In a study performed by Jaitin and collaborators, the authors engineered an IFN-α2 triple mutant (with H57A, E58A and Q61A mutations) that binds IFNAR1 with a 30-fold higher affinity than the wild-type protein and thus comparable to the binding affinity of IFN-β to IFNAR1. They observed that the HEQ mutant exhibited several functional characteristics of IFN-β, like similar patterns of gene induction and therefore substantially increased antiproliferative activity, without altering antiviral activity and ISGF3 formation. In this way, they indicated that functional differences between IFN-α2 and IFN-β are mainly due to their different binding affinities for IFNAR1. Therefore, the differential phenotypes of IFN-α2 and IFN-β are not qualitative, an observation that applies to both biological activities and gene induction patterns [47].

Also, the concentration of IFNAR1/IFNAR2 in the plasma membrane is critical to determine the cell sensitivity to type I IFNs. While IFN-β is more potent than IFN-α2 to induce antiproliferative activity in cells with native receptor numbers, IFN-α2 was equally able in cells with highly

increased receptor numbers [46]. This may explain why IFN-β, but not IFN-α2, provides long-term signaling [70]. In Daudi cells, picomolar concentrations of type I IFNs are already enough to cause growth arrest because these cells have higher levels of the interferon receptors [51].

A recent report has suggested that tumor cells have lower interferon receptor levels, making them resistant to the antiproliferative activity of type I IFNs [71]. At low IFNAR1 concentrations, the complex is formed only with IFN-β, which binds IFNAR1 tightly, but not with IFN-α2 [72]. The duration of IFN stimulus is also critical to the decision between the antiviral state or antiproliferative response. While induction of antiviral activity requires a few hours of IFN-α or IFN-β exposure, antiproliferative activity requires days of constant IFN-α/β binding [70].

Alterations in the type I interferon pathway in cancer

Since type I IFNs play a central role in the tumor microenvironment, especially with regard to anti-tumor activities, genetic alterations in this pathway are expected to be detrimental to prognosis and responses to therapy. The interferon gene cluster, found on human chromosome 9p21, encodes nearly all of the IFN genes and pseudogenes [43]. Interestingly, the cyclin dependent kinase inhibitor 2A (*CDKN2A*) gene also resides in this same region, encoding the $p14^{ARF}$ (alternate reading frame, a functional partner of p53) and $p16^{INK4a}$ (inhibitor of CDK4/6, thus an activator of retinoblastoma - Rb) proteins [73]. As such, 9p21 deletions could impact the p53, Rb and interferon pathways together or individually, depending on the exact nature of the deletion.

Homozygous deletion of IFN-α/β has been reported in leukemias, such as chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) [74–78], and may be associated with resistance to IFN-α [79]. However, other studies indicate that a 400kb deletion including $p16^{INK4a}$, but not the IFN gene cluster, is critical in lymphoblastic leukemias [80]. Deletion in the gene encoding IFN-α has also been correlated with post-transplant lymphoproliferative disorder [81]. Homozygous deletion of IFN-α contributes to the recurrence of head and neck squamous cell carcinoma (HNSC) [82]. The loss of the gene encoding IFN-β has been observed in glioma [83–85]. While for melanoma, hemizygotic deletion of 9p21 has been reported, observed in 12 of 14 cases [86], but this may reflect loss of *CDKN2A* alone or in combination with IFN-β [85]. Studies in a variety of cancer cell lines also support the notion that loss of the genes encoding IFN-α/β is a frequent event, though co-deletion of $p16^{INK4a}$ and/or $p14^{ARF}$ must be specifically examined [87–89].

Alterations in the interferon gene cluster are certainly not the only mechanism by which the type I IFN

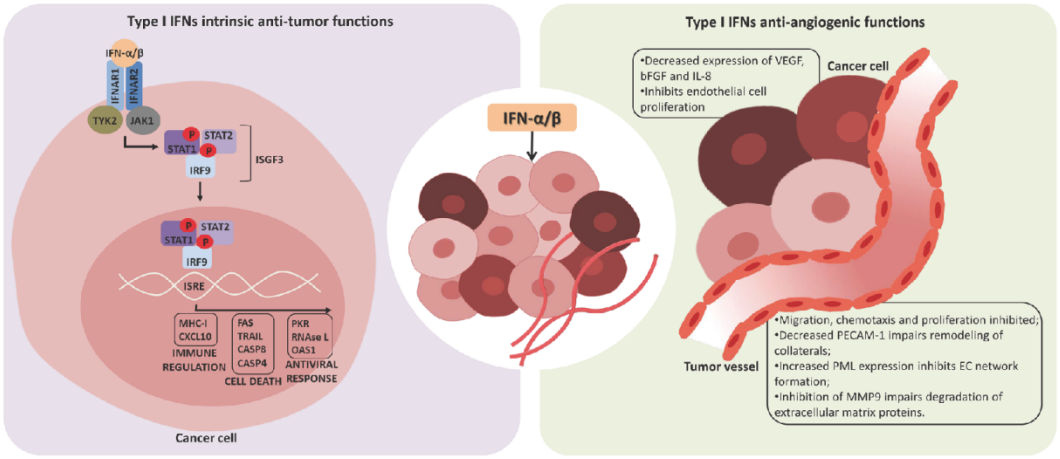

Figure 1: Intrinsic anti-tumor and anti-angiogenic functions of type I interferons. Interferon-α/β (IFN-α/β) has direct effects in tumor cells inducing growth arrest and cell death (left panel). After binding to the heterodimeric IFN-α/β receptor 1 and 2 (IFNAR1/IFNAR2), type I IFNs induce a cascade of intracellular events which culminates in expression of genes whose promoters contain the IFN-stimulated response element (ISRE). In this way, several immuno-regulatory cytokines, cell death factors and proteins related to antiviral response are produced, as well as more IFN-α/β, which in turn affects neighboring cells. In addition, anti-tumor effects of type I IFN may also be a consequence of its anti-angiogenic function, impairing tumor vessel formation and leading to death of tumors by lack of oxygen and nutrients (right panel). IFN-α/β can inhibit the production of angiogenic factors by tumor cells, and also directly affects endothelial cells (EC), inhibiting their proliferation and secretion of factors responsible for EC chemotaxis and remodeling of extracellular matrix. Tyrosine kinase 2 (TYK2), Janus kinase 1 (JAK1), signal transducer and activator of transcription (STAT), IFN-regulatory factor 9 (IRF9), IFN-stimulated gene factor 3 (ISGF3), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), platelet endothelial cell adhesion molecule-1 (PECAM-1), promyelocytic leukemia protein (PML), matrix metalloproteinase 9 (MMP9).

pathway may be disrupted. At least in cell lines, loss of STAT2 is associated with reduced apoptosis in response to IFN-α treatment [90]. Interestingly, methylthioadenosine phosphorylase (*MTAP*) also resides on chromosome 9p21 and can be epigenetically silenced in melanoma, resulting in impaired STAT1 signaling and serving as a marker of response to IFN-α therapy [91]. Infection with human papillomavirus (HPV), whether of high or low risk subtypes, has been correlated with resistance to IFN-α [92, 93]. Stimulator of interferon genes (STING) acts in the endoplasmic reticulum and promotes the transcriptional functions of nuclear factor-κB (NF-κB) and IRF3, thus playing a major role in anti-viral response [94]. In established melanoma and colorectal cancer cell lines, STING signaling is repressed due, typically, to epigenetic silencing of cyclic GMP-AMP synthase (cGAS) or STING itself. Loss of STING and/or cGAS was confirmed in some 54% of human colorectal cancer (Stage II-IV) while loss of both was seen in 41.7% of advanced stage human melanoma samples [95, 96].

Type I IFN and its receptor (IFNAR1/2) also contribute to the immunosuppressive tumor microenvironment. The paradox between inflammation and immunosuppression, especially with regard to type I IFN, was reviewed recently [97]. Expression of type I IFN is expected to promote the immune response, but it can also lead to the expression of indoleamine 2,3-dioxygenase (IDO), programmed cell death-ligand 1 (PD-L1) and IL-10, culminating in immunosuppression, as discussed here and in the review by Snell et al. [97]. In a recent report, Katlinski et al. [98] showed that IFNAR1 expression is reduced in colorectal cancer microenvironment, specifically in cytotoxic T lymphocytes, contributing to an immune-privileged niche that supports tumor growth. Restoration of IFNAR1 expression in the T cells was associated with renewed control over tumor growth [98]. The magnitude and duration of type I IFN signaling may be critical points to consider when designing therapies aimed at this complex pathway since sustained inflammation may lead to immunosuppression.

ANTITUMOR FUNCTIONS OF TYPE I INTERFERONS

Inhibition of cell growth and induction of apoptosis

The effects of type I IFNs on tumor cells may vary depending on the type of tumor and even more so when considering the specific cell in question. For example, IFN-β has a stronger antitumor effect than IFN-α in the early stage hepatocelular carcinoma (HCC) in patients with chronic hepatitis C. While IFN-α has been shown to induce apoptosis in HCC cell lines [99–101], Murata and co-authors showed that IFN-β had a superior antiproliferative effect as compared to IFN-α on three HCC cell lines, inducing cell cycle change and apoptosis, and more strongly upregulating ISGs, like Fas antigen and human leukocyte antigen (HLA)-class I molecules [102].

A time course study in WM9 melanoma cells with IFN-β (500 units/ml) identified more than 30% terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells at 96 h, while IFN-α2 did not result in any positive staining. Other melanoma cell lines revealed similar sensitivity, like FemX cells, while Guilliams cells were partially sensitive and A375 cells were relatively resistant to either IFN-α2 or IFNβ, even at higher doses (up to 1000 units/ml) [103]. In another study with melanoma cells, IFN-β potency was also greater than IFN-α2 for induction of ISGs, like cytomegalovirus-induced gene 5 (*CIG5*/*viperin*), *CIG49*, *ISG54*, *TLR3*, *CXCL10*, *TRAIL*, as seen by microarray analysis of both WM9 and WM35 cell lines, while for the IFN-β-sensitive WM9 cell line, IFN-β also induced expression of *SP100*, tumor necrosis factor-stimulated gene 6 (*TSG6*), augmented in prostate carcinoma gene (*AIPC*), *Cyclin-E*, ubiquitin E2-like (*UBEL-2*) and ubiquitin-specific protease (*USP18*), as seen by RT-qPCR [104].

SK-MEL-2 and SK-MEL-24 cell lines were also more sensitive to the anti-proliferation effects of IFN-β than those of IFN-α2b *in vitro*. Matrigel invasion of SK-MEL-24 was significantly inhibited by both IFN-α2b and IFN-β and treatment of SK-MEL-24 with IFN-α2b or IFN-β decreased vascular endothelial growth factor-C (VEGF-C) and VEGF receptor-3 (VEGFR-3) protein expression. In a human melanoma xenograft model, SK-MEL-24 cells were injected intradermally in mice and tumor growth was reduced after IFN-α2b or IFN-β treatment. LN metastases were more frequent in mice treated with IFN-β than with IFN-α2b. One of six mice showed LN metastasis in the IFN-α2b group compared to three of six mice in the IFN-β group. Tumors were evaluated and revealed that both IFN-α2b and IFN-β decreased cell proliferation and increased the number of apoptotic cells, yet these effects were superior in IFN-β treated tumors. Also, VEGF-C/VEGFR-3 levels were reduced in tumors treated with IFN-α2b or IFN-β, but LYVE-1 was decreased only in IFN-α2b treated tumors, representing less intratumoral and peritumoral lymphatic vessels [105].

Cell lines that were relatively resistant to inhibition of cell growth by IFNs, including U937 (histiocytic lymphoma), HeLa (HPV-infected cervical adenocarcinoma), and T47D (ductal breast carcinoma) were also not TUNEL-positive in response to IFN-α2 or IFN-β. Other cell lines, like ACHN (renal cell carcinoma), Minors (melanoma), NIHOVCAR3 (ovarian carcinoma) and MCF-7 (breast carcinoma) had an increase in TUNEL-positive cells in response to IFN-β but not IFN-α2 [103].

Chen and colleagues showed that both IFN-α and IFN-β induced apoptosis in U266, RPMI-8266, and NCI-H929 multiple myeloma cell lines and plasma cells from 10 patients with multiple myeloma [106]. Expression of TRAIL, which contains 2 IFN-stimulated regulatory

elements in its promoter [107], seems to be the main event for cell death induction, followed by caspase-8 activation, Bid cleavage, cyt c release and caspase-3 activation [106].

In a study performed by Rozera and co-authors, TS/A adenocarcinoma cells were injected with retrovirus encoding IFN-α or IFN-β and these cells were inoculated in BALB/c mice. More host-infiltrating cells were observed in TS/A-IFN-α and TS/A-IFN-β than in parental TS/A tumors (macrophages, granulocytes, and lymphocytes, being $CD8^+$ T cells more numerous than $CD4^+$ T cells). Also, fewer blood vessels were observed in TS/A-IFN-α or TS/A-IFN-β tumors as compared with parental TS/A tumors, being the vasculature of TS/A-IFN-β tumors scarcer than TS/A-IFN-α tumors, even when no differences in the expression of angiogenic factors (VEGF and basic fibroblast growth factor - bFGF) were found. However, the expression of pro-inflammatory cytokines, such as IL-1β, IFN-γ and tumor necrosis factor-α (TNF-α), was higher in TS/A-IFN-α than TS/A-IFN-β tumors and absent parental TS/A tumor. Finally, survival of TS/A-IFN-β mice that produced higher levels of IFN-β or TS/A-IFN-α mice was three- to four fold longer than the control group, while metastatic ability of TS/A cells was reduced in mice injected with either TS/A IFN-α or IFN-β cells [108].

Inhibition of angiogenesis

Angiogenesis is an important antitumor therapeutic target because it is required for tumor growth [109, 110] for the delivery of oxygen and nutrients to the fast-growing tumor cells [111]. As shown in Figure 1, the anti-proliferative and cell death inducing functions of type I IFNs also inhibit angiogenesis [112, 113]. For example, after IFN-α/β treatment, tumor vessels undergo necrosis [113]. IFN-α/β also prevents tumor cell production of angiogenic growth factors, like bFGF [114–116], VEGF [117, 118], and IL-8 [119, 120]. Interestingly, when MBT-2 (murine transitional carcinoma of the bladder) or L1210R (murine leukemia resistant to the antiproliferative effects of IFN) cells were treated *in vitro* with IFN-β and then inoculated intracutaneously in C3H/He or Swiss mice, respectively, the inhibition of angiogenesis was noted within 24 hours of tumor cell inoculation, even before their antiproliferative effects on tumor cells [112]. Thus, inhibition of angiogenesis can be counted among type I IFN's anti-tumor benefits.

McCarty and collaborators showed that endogenous type I IFN signaling is involved in the regulation of angiogenesis. These authors implanted sponges filled with proangiogenic molecules (bFGF, VEGF, and TGF-α) in mice deficient for IFN-α/β receptor and observed superior vascularization when compared to control mice with functional type I IFN signaling. Moreover, the antiangiogenic effects of type I IFNs resulted in inhibition of tumor growth in animal models [121].

It is known that in inflamed tissues, the release of IFN-α by leukocytes trigger macrophage activation, which then causes the release of TNFα. Both IFN-α and TNF-α induce accumulation of promyelocytic leukemia protein (PML) in HUVECs and in microvascular endothelial cells (HMVECs). PML was shown to be indispensable for TNF-α and IFN-α-mediated inhibition of EC network formation, but no significant differences in apoptosis of HUVEC treated with TNF-α, IFN-α or vehicle were detected [122]. PML is highly expressed in normal vascular endothelium and inflamed tissues [123] and known as an ISG [124] through STAT1 induction, since knockdown of STAT1 significantly impairs PML expression in these cells. The authors have shown PML suppressed integrin β1 (ITGB1) expression in both HUVECs and HMVECs, an important protein which regulates ischemic neovascularization [125], cell-to-cell and cell-to-extracellular matrix adhesion and cell migration [126].

Spaapen and others have observed that IFN-β–secreting B16 cells injected subcutaneously in mice had impaired growth, yet when implanted into mice lacking IFNAR1, tumors grew progressively. This shows that the antitumor effect of IFN-β was dependent on signaling via host cells and did not act directly on tumor cells, a situation that was also observed with IFN-α. This effect was also independent of adaptive immunity, since tumor regression was also observed in $Rag2^{-/-}\gamma c^{-/-}$ mice (deficient in T, B, and NK cells). However, a diminution of blood vessel density was observed in the IFN-β-secreting tumors. In the work, the authors showed that IFN-β has a direct effect on nonhematopoietic $Tie2^+$ cells, that is to say, vascular ECs, causing inhibition of angiogenesis [127].

While IFN-β may reduce the number of tumor vessels, it can also contribute to vessel maturation. Dickson and others showed that treatment of human xenografts in immunodeficient mice with an adeno-associated virus (AAV)-vector encoding the human IFN-β gene resulted in maturation of the intra-tumor vasculature, yet inhibition of angiogenesis [128]. Also, treatment of tumors with IFN-β, encoded by an adenoviral vector, promotes an increase in inducible nitric oxide synthase (iNOS) and a decrease in bFGF and TGF-β1 levels [129], resulting in inhibition of tumor growth.

Indirectly, IFN-β inhibits matrix metalloproteinase 9 (MMP9) gene expression, which is responsible, together with MMP2, for degradation of extracellular matrix proteins collagen and elastin, a process required for initiating the enlargement of collateral vessels [130]. This is in accordance with Nelissen and others (2002), who have shown that IFN-β inhibited expression of MMP9 in monocytic and peripheral blood mononuclear cells [131] (Figure 1).

The mechanisms by which IFN-β inhibits tumor growth and angiogenesis may also involve tumor-infiltrating neutrophils. Jablonska and others showed that IFN-β-deficient mice presented faster tumor growth of injected tumor cells and larger tumors compared to

wild-type mice. This was associated with an increase in tumor angiogenesis and tumor-infiltrating $CD11b^{+}Gr1^{+}$ neutrophils, which are responsible for expression of proangiogenic and homing factors. After treatment with IFN-β, these neutrophils had reduced gene expression of *VEGF*, *MMP9*, CX-C chemokine receptor type 4 (*CXCR4*) and the receptor for stromal-derived factor-1 (*SDF-1*), contributing to limiting tumor angiogenesis. Also, when neutrophils obtained from IFNAR-deficient mice were injected in wild-type mice, tumor growth was increased and accompanied by more mature vessels when compared with neutrophils obtained from WT mice [132].

Type I IFN may affect endothelial cell (EC) survival [133] and function, as indicated by inhibition of migration, chemotaxis and proliferation of HUVEC (human umbilical vein endothelial cells) [134], but as said before, although these cytokines share the same receptors, they exert different effects on tumor viability and angiogenesis. While there are several studies demonstrating the antiangiogenic effects of type I IFNs, there are few studies comparing the different effects of IFN-α and IFN-β, and even less including other members of the type I IFN family. In a study comparing type I IFNs, IFN-α2b inhibited *in vitro* vessel formation of HUVEC by 20%, whereas inhibition due to IFN-β was around 80%. *In vivo*, IFN-α2 inhibited vessel growth by 30% in SK-MEL-1 tumors, whereas IFN-β inhibited vessel formation by 80%. While both IFN-α2b and IFN-β inhibited HUVEC proliferation, neither of them was able to induce apoptosis. Genes induced by IFN-β in HUVECs are *p56*, *CXCL11*, *ISG20*, melanoma differentiation-associated-5 (*MDA-5*), HECT and RLD domain containing E3 ubiquitin protein ligase family member 6 (*HERC6*), *CXCL10*, SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (*SAMHD1*), *p60*, *Sp100B* and monocyte chemoattractant protein 2 (*MCP-2*) [135]. This effect was also observed by Erdmann and others who described that IFN-β inhibited cell cycle and proliferation of human micro and macrovascular ECs, but did not induce apoptosis [136]. Interestingly, VEGF is responsible for phosphorylation of IFNAR1, followed by ubiquitination induced by the protein kinase D2 (PKD2), which results in the degradation of IFNAR1 and promotion of angiogenesis [137].

Albini and co-authors have compared the effects of retroviral vector-packaging cell lines encoding IFN-α or IFN-β cDNAs (α1Am12 and βAm12) upon ECs. In this study, both conditioned media from α1Am12 or βAm12 decreased chemotaxis and invasion of ECs, however only βAm12 inhibited EC differentiation into capillary-like structures on Matrigel. Also, IFN-β's superiority in inhibiting angiogenesis was confirmed in an *in vivo* model, in which sponges containing a very potent angiogenic cocktail co-injected along with α1Am12 cells in C57BL/6 mice produced a limited inhibitory effect on angiogenesis, while βAm12 cells markedly impaired vessel formation. This was also observed in nude mice, confirming the effect of IFN-β on ECs independent of a T-cell response [138].

As will be discussed below, the first antitumor efforts involving type I IFNs were developed using IFN-α, but IFN-β later gained importance in this field as a result of studies showing its increased antitumor and antiangiogenic effects, although further investigation is needed to support the notion that IFN-β provides superior antiangiogenic activity. Studies comparing the gene expression profiles of ECs treated with different type I IFNs could be very enlightening as to the different effects of these cytokines on angiogenesis. Also, *in vivo* and *in vitro* treatments of ECs with different type I IFNs and posterior analysis of angiogenesis could reveal type I IFN's functional effects. Finally, knockout models specific for each type I IFN could be used to confirm their individual functions, thus providing evidence based on endogenous proteins.

Immunomodulatory and regulatory effects of type I interferons

So far, we have exposed several anti-neoplastic functions attributed to type I IFN, but its main function is most often associated with immune modulation. Depending on the stimulus, both IFN-α/β can be produced by almost any cell type, including fibroblasts and leucocytes [139]. Such inducers act through pattern recognition receptors (PRRs) that sense pathogen-derived and non-pathogenic components, for example: double-stranded RNA (dsRNA) from RNA viruses detected by TLR3, both cytosolic DNA and second messenger cyclic di-GMP24 from bacteria by STING [5, 139], as well as danger-associated molecular patterns (DAMPs) released upon cellular stress or therapy induced cell death. Heil and Land suggest dividing mammalian DAMPs into five classes according to different PRRs. Class I DAMPs, like HMGB1 or heat shock proteins, are sensed by TLRs and trigger the MAPK signaling cascade. Class II DAMPs are perceived indirectly by the NOD-like receptor family protein 3 (NLRP3) inflammasome and comprise ROS, monosodium ureate, eATP and dsDNA. Both classes I and II are involved in maturation of DCs. Class III comprises stress-induced soluble major histocompatibility complex class I-related chains A/B (MIC-A/B) and UL-binding proteins (ULBPs) and are sensed by receptors such as NKG2D, expressed by innate lymphocytes, like NK cells, and innate-like T-lymphocytes, like gamma delta T-cells. Class IV represents neoantigens, such as non-muscle myosin-II (NMHC-II), actin cytoskeleton and oxidized phospholipids that, together with IgM antibodies, bind to classical lectin receptors and trigger activation of the complement cascade and alternative pathways. Finally, class V DAMPs are called Dyshomeostasis – Associated Molecular Patterns, which comprise altered pattern of molecules resulted from perturbations in the steady-state of the intra- and/or extracellular microenvironment, like hypoxia, changes in acidity or osmolarity, and metabolic

stress [140]. Thus, type I IFNs may be released as part of the natural evolution of a disease or as a consequence of therapeutic interventions. However, in cancers, an important difference between these two scenarios is that the local immune suppressive environment modifies the expected "physiological" response, either counteracting or amplifying the immunomodulatory functions of type I IFNs (Figure 2).

Immunomodulatory effects triggered by type I IFNs can act on both innate and adaptive immune compartments [141]. In a temporal scale, macrophages can be considered as early producers of type I IFNs that act on nearby macrophages and other innate cells, such as NK, to provide a pro-inflammatory context (release of cytokines IL-6, TNF-α) suitable for antigen capture and presentation by tumor associated APCs and priming of immune effector cells [2, 142]. Macrophages, similarly to DCs, produce both type I and II IFNs, and upon activation display high levels of MHC class I and II in order to boost a T cell response, thus working as a link between innate and adaptive immunity [143]. Type I and II IFNs have also been show to polarize macrophages into an M1 immunostimulatory phenotype with anti-tumor functions, rather than an M2 phenotype, which may have pro-tumor activities [143, 144].

However, as part of an adaptive immune resistance mechanism that takes place after an inflammatory response, negative regulators of the immune response are induced, aiming to limit both duration and specificity of the immune attack [145]. Regulatory mechanisms induced after IFN-α/β production include secretion of IL-10 and expression of PD-L1 [146]. Both IL-10 and PD-L1 are well known inhibitors of $CD8^+$ T function and, as shown by Shaabani and collaborators, upon infection with lymphocytic choriomeningitis virus (LCMV), type I IFNs are produced in high amounts by $CD169^+$ macrophages to combat the virus, but as a consequence leads to up-regulation of PD-L1 and therefore $CD8^+$ T cell exhaustion [147]. Lack of this $CD169^+$ macrophage population, known for their unique distribution in secondary lymphoid organs and antigen handling capacity to prime $CD8^+$ T cells, impairs viral control, IFN-α production, and eventually mice succumb, thus confirming the regulatory function type I IFNs on macrophages and $CD8^+$ responses [147, 148]. Interestingly, in a more general view of the tumor microenvironment context, PD-L1 expressing tumor-infiltrating immune cells, including macrophages and DCs, in head and neck cancer patients have been shown to result in a more favorable prognosis than when PD-L1 is expressed on tumor cells [149]. Indeed, in a recent study by Noguchi and colleagues, IFN-γ was responsible for up regulating PD-L1 in tumor cells, contributing to immune escape, but unexpectedly, the majority of PD-L1

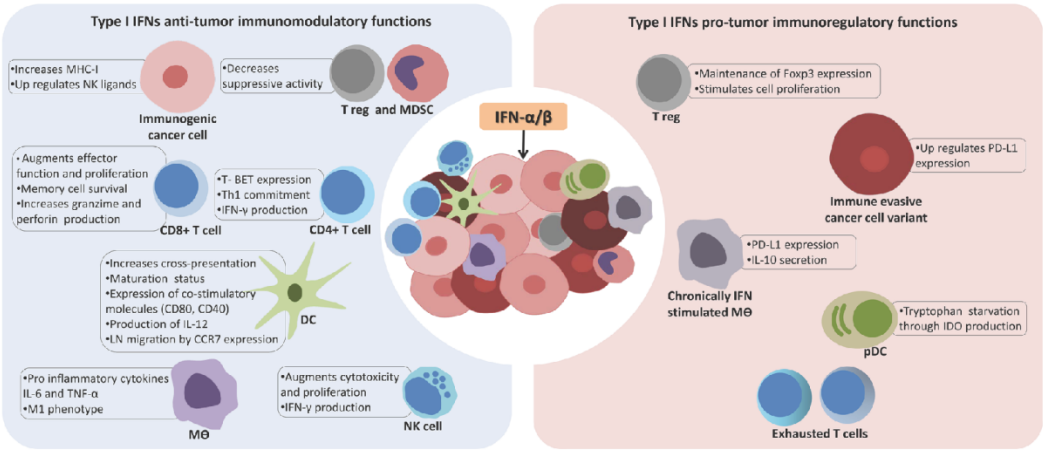

Figure 2: The context dependent and complex role of type I interferons in cancer immunity. Activation or delivery of interferon-α/β (IFN-α/β) into the tumor microenvironment can result in immunomodulatory and regulatory functions. In the first scenario (left panel), in order to unleash an effective immune attack against cancer cells, type I IFNs modulate innate and adaptive compartments through multiple mechanisms to provide a pro-inflammatory context suitable for antigen recognition by tumor associated dendritic cells (DCs) and priming of T lymphocytes. Importantly, as type I IFNs enhance co-stimulatory molecules of DCs, they also increase the unique ability of DCs to cross-present phagocytized tumor antigens to $CD8^+$ T cells. Additionally, immunogenic tumor clones (represented in pink) have their antigenicity increased by up regulating major histocompatibility complex class I (MHC-I) molecules. However, type I IFNs can also favor tumor progression and escape from immune control (right panel), especially under chronic exposure conditions, since they can induce macrophages (mΘ) to produce Interleukin-10 (IL-10), that along with tryptophan starvation mediated by indoleamine 2,3-dioxygenase (IDO) and expression of programmed death-ligand 1 (PD-L1) by immune evasive tumor cells (dark red), greatly impairs T cells functions. Tumor necrosis factor–α (TNF-α), plamacytoid DCs (pDCs), myeloid-derived suppressor cells (MDSC), regulatory T cell (T reg), C-C chemokine receptor type 7 (CCR7), natural killer cells (NK).

molecules were expressed by the host immune system, especially in the macrophage compartment, suggesting a mechanism in *trans* to impair T cells function. Intriguingly, after antibody mediated blockade of IFN-γ, levels of PD-L1 on tumor cells drastically decreased, but remained elevated on tumor associated macrophages, suggesting an additional mechanism not dependent on IFN-γ to induce PD-L1 [150]. The exact role for type I IFNs in mediating PD-L1 immune escape remains to be elucidated, but it is tempting to speculate that early production of IFN-α by APCs augments IFN-γ production by $CD4^+$ T lymphocytes [151] and NK cells [152], and thus leads to PD-L1 up regulation in the tumor microenvironment.

Within the DC compartment, type I IFNs act as strong maturation signals, increasing expression of co-stimulatory proteins CD40, CD80, CD86 and MHC molecules [153], enhancing their unique properties to process and present apoptotic cell antigens through their scavenger receptor lectin-like oxidized-LDL receptor-1 (LOX-1) [154], and stimulating migration to the draining LNs by up regulating the CCR7 chemokine receptor [155]. Notably, together with the production of IL-12 and IL-23, all of these actions support Th1 and Th17 cytotoxic T lymphocyte (CTL) responses, including increased survival of $CD8^+$ memory T cells and expression of granzyme B and perforin 1 [2, 5].

Among the different DC subsets, using bone marrow chimera experiments, Diamond and collaborators have demonstrated that *Ifnar1*$^{-/-}$ $CD8\alpha^+$ DCs lose their capability to cross present tumor antigens and as a consequence, when regressor tumors are transplanted, the host is no longer able to immune reject them [9]. Cross-presentation is defined as the presentation of internalized antigens in the context of MHC-I molecules to $CD8^+$ T cells, instead of the MHC-II context [5]. This data implies that this DC subset must be endowed with mechanisms to recognize stress signals from dying tumor cells, capture their antigens and present to $CD8^+$ T cells, in order to trigger CTL responses. A plausible mechanism for the re-routing of capture antigens has been made by Reis and Sousa, investigating the C-type lectin domain family 9 member A (CLEC9A), a plasmatic receptor for necrotic cells that is highly expressed in CD8α DCs. They found that instead of activating these cells, CLEC9A was directing necrotic cell cargo into a recycling endosomal compartment, favoring cross-presentation to $CD8^+$ T cells. Along the same lines, CLEC9A deficiency in $CD8\alpha^+$ DCs impaired their capability to prime $CD8^+$ T cells but not $CD4^+$ T cells, indicating that this plasmatic receptor was promoting cross-presentation of dead cell–associated antigens on MHC class I molecules. However the role of CLEC9A on the cancer immunity cycle needs further investigation [156]. Along the same lines, *Tmem173*$^{-/-}$ DCs, that lack the STING pattern recognition receptor gene, have also been found to be unable to prime $CD8^+$ T cells [5, 157].

Taken together these data indicate that type I IFNs are involved in DCs antigen preseting functions, although immune regulatory mechanism are also triggered in order to limit the magnitude of the inflammatory response. Evidence of such regulatory mechanisms can be observed in pDCs, which have an unclear role in cancer, but are known to be a major source of type I IFNs. pDCs that infiltrate breast cancers have been shown to be defective in producing IFN-α and to co-localize with T regulatory cells (T-reg, $CD3^+CD4^+CD25^+FOXP3^+$) [158], suggesting that either T-reg cells may be inhibiting IFN-α production or that pDCs somehow support the proliferation of T-regs. Indeed, mature pDCs can orchestrate tolerogenic immune responses through the induction of IL-10 IDO [159, 160], which catabolizes the essential amino acid tryptophan into a more stable metabolite, kynurenine and, as a consequence, stimulates T-reg proliferation while inducing $CD8^+$ T-cell dysfunction, anergy and apoptosis [160, 161]. Catabolism of tryptophan in cancer is being recognized as a powerful suppressor of antitumor immunity since several tumor types were found to over-express IDO [162] and this was recently implicated as a critical mechanism of resistance to checkpoint blockade immunotherapy targeting the cytotoxic T lymphocyte antigen-4 (CTLA-4) pathway [163]. Interestingly, the IDO promoter contains transcriptional targets of both IFN-γ and IFN-α/β and in the case of pDC, both type I and type II IFNs were shown to be equipotent and exert additive effects on the induction of IDO [164].

In the lymphocyte compartment, type I IFNs have been show to act on both $CD4^+$ and $CD8^+$ T cells to dictate a Th1 immune response through the activation of STAT4 and T-bet expression, which is a T-box transcription factor expressed in $CD4^+$ T lymphocytes committed to Th1 development [165, 166]. Interestingly, type I IFNs can also reverse the commitment of a Th2 humoral response by suppressing the GATA3 transcription factor, but when compared to IL-12, another Th1 inducing cytokine, it was shown that type I IFN cannot sustain T-bet expression by itself, needing other cytokines, such as IL-1β, to maintain this phenotype [166]. In terms of cancer, Th1 cells are known for orchestrating CTL responses that are implicated in the destruction of a tissue during autoimmune responses as well in antitumor responses [167], as demonstrated by a mechanistic degree of similarity shared between them [167, 168] and increased survival rate observed in patients with a CTL tumor infiltrate [169].

Another critical immunomodulatory mechanism induced by type I IFNs that directly affects T cell responses is the positive regulation of tumor antigens that are presented on cancer cells by MHC-I molecules, allowing the immune system to detect the tumor and distinguish it from a normal cell [170, 171]. In fact, up regulation of MHC-I by type I IFNs [172], has the potential to counteract the frequent down regulation of MHC-I found in human tumors resistant to immunotherapies [173].

Indeed, as recently demonstrated in a mouse model using tumors resistant to PD-L1 checkpoint blockade immunotherapy, high dose radiation directly upon the tumor mass induces a systemic increase of IFN-β levels and restores therapeutic efficacy by up-regulating MHC-I molecules in the tumor cells. Antibody mediated blockade of IFNAR1 completely abrogated this effect [174].

Furthermore, the regulation of immunity in cancer by IFN-α/β also involves increased cytotoxic functions of NK cells [146], which are lymphoid cells that through a balance of activating (e.g., NKG2D) and inhibitory receptors (e.g, killer-cell immunoglobulin-like, KIR) can effectively kill tumor cells [175]. Activation of the NK response was demonstrated to positively sustain an M1 macrophage phenotype and to edit tumor immunogenicity in a process independent of T cells [176]. And, curiously, type I IFNs inhibit the elimination of $CD8^+$ T cells by NK cells [177], a phenomenon observed in conditions where NK cells assume a regulatory function over the adaptive immune response in order to prevent chronic inflammation and generation of auto-immune reactions [178].

Finally, in support of defining the appropriate context for an effective CTL response, type I IFNs were also shown to inhibit immune-suppressive actions of T-reg cells and MDSCs [144]. Yet in inflammatory conditions, it has been shown that type I IFNs were required for maintenance of Foxp3 expression and immune suppressive functions of T-regs, since transfer of T-regs obtained from *Ifnar1* KO mice were not able to inhibit the induction of the T-cell mediated colitis, as seen for T-regs obtained from wild-type mice. Indeed, administration of recombinant IFN-α reduced T cell-mediated colitis by increasing the number of T-regs and their suppressive functions [179].

As discussed here, the role of type I IFNs in immunity is complex and context dependent, assuming either antitumor or pro-tumor functions determined by the exposure to type I IFNs pre or post antigen encounter, if produced acutely or chronically, in low or high levels. For example, using an LCMV model, it was observed that early and transient production of IFN-α by pDCS exerts minimal effects on $CD8^+$ T cell responses, but administration of recombinant IFN-α5 and IFN-β on days that coincide with endogenous type I IFNs decline, hence providing sustained stimulation, can prevent $CD8^+$ T cell exhaustion and viral persistence [180]. Furthermore, this complex role of type I IFNs was evidenced in two complementary works both exploring a model of chronic LCMV infection, where IFN-α was transiently produced, yet ISG expression was prolonged. In the first work, it was shown that genetic or antibody mediated blockade of IFN-α signaling prior to infection leads to increased viral replication and loss of infection control, thus confirming the antiviral role of IFN-α [181]. Whereas, in the second, after establishment of the chronic infection, IFN-α blockade acted by reducing IL-10 and PD-L1 levels and, as a result, ameliorated T cell exhaustion and, even though it took 2 months, resulted in significantly lower virus titers [182]. Therefore, there seems to be a paradoxical function of type I IFNs: early (i.e., prior to antigen encounter) antiviral effects of type I IFNs are critical for host protection, promoting immune activation by stimulating an NK cell attack, enhancing DC antigen presenting function and favoring T cell proliferation, but after this adaptive immune response has been unleashed, chronic stimulation of the type I IFN pathway can result in immunoregulatory mechanisms that aim to shut down long lasting and unresolved immune responses, although as discussed below, therapeutically induced IFN-α/β can restart or reinvigorate a new immunity cycle. Examples of such duality are also observed during hepatitis C virus infection, in which strong IFN-α/β signature correlates with poor responses to therapy, as well as in chronic HIV infections and on latent *Mycobacterium tuberculosis* [183].

In cancer, the opposing role IFNs is better characterized with IFN-γ, especially on the induction of PD-L1 on cancer cells. As recently demonstrated by Benci and colleagues, prolonged exposure of tumors to IFN-γ induces a STAT-1 epigenetic signature as well as ligands for inhibitory receptors that results in PD-L1 dependent and independent mechanisms of resistance to checkpoint blockade immunotherapy [184]. Intriguingly, type I IFNs were also shown to be required for maintenance, not induction, of the PD-L1 independent resistance phenotype, but the precise contribution was not thoroughly explored [184]. Moreover, disrupting IFN-γ driven resistance with ruxolitinib, a JAK1/JAK2 inhibitor, renders CTLA-4 checkpoint blockade resistant tumors sensitive again. Accordingly, the work also provides clinical evidence that high expression of ISG and IFN-γ signaling is associated with tumor progression after PD-L1 therapy [184]. The mechanism that is behind the complex and opposing functions of IFNs is likely mediated by a qualitative and quantitative difference of regulators. For example, in a mouse melanoma model, therapeutic efficacy of high-doses of intratumoral IFN-α/β appears not to be T cell dependent, but rather relies on their anti-angiogenic properties, acting directly on the tumor vasculature [127]. Further studies that can dissect the molecular basis of this complex mechanism, specifically the influence of the producing cell, timing and magnitude, are surely needed.

TYPE I INTERFERONS IN CANCER THERAPY

IFN-α/β therapy for melanoma

Type I IFN therapy as treatment for melanoma utilizes the recombinant protein itself or as a complex with polyethylene glycol (PEG) in order to improve protein

stability [185]. High dose IFN-α2b has been approved as an adjuvant therapy after surgical resection of cutaneous melanoma in patients with a high risk of death from recurrence. This approach is beneficial for improving disease-free survival, but the therapy itself is not well tolerated. Here we will address some of the progress and pitfalls of IFN-α/β therapy.

When caught early, surgical excision of melanoma can be curative. However, once disseminated, the treatment of melanoma is quite inefficient and survival rates are quite dismal [186]. The use of IFN-α2b was approved as an adjuvant therapy by the FDA in 1996 based on clinical findings that showed high-dose treatment was beneficial for prolonging relapse free survival and overall survival [187]. Unfortunately, the high-dose treatment is associated with severe adverse effects, including fatigue, myalgia, pyrexia and depression. While lower doses may decrease the adverse effects, they do not offer the same benefit in relapse free survival [188]. The use of PEG-IFN-α2b has been shown to reduce some of the fatigue and flu-like symptoms seen with the non-pegylated protein [189, 190]. In comparison, adjuvant therapy with IFN-β is standard practice in Japan where low-dose administration has been reported as beneficial for maintenance therapy [191].

High-dose IFN-α treatment has also been tested as a neoadjuvant for patients with locally advanced disease with the intention of reducing T-regs and improving $CD8^+$ T cell memory [192]. Clinically, neoadjuvant IFN-α therapy was associated with increased intratumoral DC in 11/20 patients who showed objective clinical response [193]. For the treatment of disseminated melanoma, higher doses or continuous administration of IFN-β were met with limited efficacy and toxicity [194, 195]. Association of IFN-α with dacarbazine or other chemotherapies was not beneficial [192].

The results from several long term and large cohort trials exploring melanoma patient populations and treatment regimens have been published in the past few years. For example, in the Sunbelt Melanoma Trial, started in 1997, treatment of patients with sentinel lymph node involvement were treated with high dose IFN-α2b (HDI) with or without complete lymph node dissection and clinical progress was compared to patients who did not receive HDI. In this trial involving more than 900 patients, no clinical benefit was associated with the use of HDI [196]. Long term follow up of the EORTC 18952 trial was recently reported, revealing that a 13 month IFN-α2b treatment regimen was inferior to a 25 month regimen in patients with stage IIB-III melanoma, however the difference was marginal. Interestingly, ulceration of the primary tumor was associated with increased sensitivity to IFN-α2b [197]. Final analysis of the Dermatologic Cooperative Oncology Group Trial was reported in 2015, showing benefit of IFN-α2b treatment for relapse free survival, but not overall survival [198].

The study of melanoma treatment using recombinant type I IFN is ongoing and aims to identify patient populations that will benefit from this and other adjuvant approaches, including ipilimumab and vemurafenib [186, 197, 199–201]. Even so, the use of type I IFN for the treatment of cancer is certainly not limited to melanoma. For example, treatment of prostate carcinoma [202] and myeloproliferative disorders [203] with type I IFN has revealed some benefit, yet concerns over decline in quality of life and the toxicity of the treatment continue. As evidenced by the large number of clinical trials involving type I interferon for the treatment of cancer (more than 450 listed on https://clinicaltrials.gov, including some 50 trials that are recruiting patients at this time), study of this approach continues in order to better develop delivery methods, treatment regimens and identify those patients who are most likely to benefit.

Inducers of endogenous type I IFNs

The main purpose of cancer immunotherapy is to induce immune cells to effectively eliminate tumors, overcoming the immunosuppressive tumor microenvironment [204] and, as discussed here, the induction of type I IFNs may be a critical step towards this end. Indeed, in contrast with traditional vaccine adjuvants, such as aluminum compounds, that mostly stimulate humoral immune responses, targeting of DAMPs and/or PAMPs receptors to induce IFN-α/β is a very effective strategy for cell-mediated immunity and therefore an alternative as adjuvant in cancer vaccines (Figure 3). Here we explore the induction of endogenous type I IFN both as an adjuvant and as an immunotherapy on its own.

Poly(I:C)

Polyribosinic-polyribocytidylic acid [Poly(I:C)] is a synthetic analog of double-stranded RNA, a ligand of the TRIF-dependent toll-like receptor-3 (TLR3) [205]. TLR3 is highly expressed in several tumors [206] and in immature myeloid DC, NK cells, T cells and macrophages [207, 208]. Poly-ICLC is a derivative of Poly(I:C) stabilized with poly-lysine (Hiltonol®, Oncovir Inc.) and indeed was shown to be 5- to 10-fold more resistant to hydrolysis [209]. Both are included in the National Cancer Institute's ranking of immunotherapeutic agents with the highest potential of improving the cancer immunotherapy response [205].

After stimulation, TLR3 recruits adaptor protein TRIF and signals through activation of IRF3, NF-κB and activator protein-1 (AP-1), stimulating activation of the antiviral and pro-inflammatory responses [210].

In vitro studies have shown that Poly(I:C) induces maturation and activation of DCs, including enhancing cross-presentation [211], stimulates T cells [212] and NK cells [213], and induces secretion of pro-inflammatory cytokines by tumor and immune cells [214]. Poly(I:C) can

also directly affect tumor growth and induce apoptosis of tumor cells [215], resulting in the availability of tumor-associated antigens (TAAs) for uptake by APCs.

In healthy volunteers, Poly-ICLC was shown to upregulate genes involved in the innate immune response including IFN-α, IFN-β, IFN-γ, the complement system and the inflammasome [216]. Poly-ICLC stimulated Th1 cytokines, increasing the Th1/Th2 ratio [217] and driving T cells toward a Th1 response. Sabbatini and colleagues have shown that 91% of patients with ovarian cancer in a poly-ICLC-vaccine cohort showed functional $CD8^+$ T cell responses, compared to 25–62% of patients in non-poly-ICLC groups. Similarly, $CD4^+$ T cell responses were stronger in patients treated with poly-ICLC-containing vaccines [218].

Interestingly, lymphocytes extracted from patients with persistent HPV infection were exposed to HPV16 virus-like particles (VLP) and then treated with Poly-ICLC, resulting in increased MHC class I and II, CD40, CD80, and CD86 expression and inducing HPV16 E7-specific $CD8^+$ T cell responses *in vitro* [219].

Another version of Poly(I:C), Poly(I:C12U) (Ampligen®, Hemispherx Biopharma), has been used for chronic fatigue syndrome treatment [220] and activated moDCs, increasing the expression of surface MHC class I and II, CD83, CCR7, CD86, CD40 and IL-12 [221].

Lipopolysaccharide

Lipopolysaccharides (LPS) from Gram-negative bacteria are TLR4 agonists. After LPS binding, TLR4 dimerizes and this is sensed by an adaptor molecule called toll/interleukin-1 receptor domain-containing adapter protein (TIRAP) [222]. Then, TIRAP recruits the signaling adaptor MyD88 and several interleukin-1 receptor-associated kinase (IRAK) family members [223], which activates inflammatory transcription factors such as AP-1 and NF-κB [224]. At the same time that TLR4 signaling is induced, several events take place to promote the TLR4 endocytosis. Upon delivery to endosomes, TLR4 recruits TRIF-related adaptor molecule (TRAM) and TRIF, which signals through a cascade of activated proteins in the cytosol and culminates in the induction of and IRF3 [225]. In this way, TLR4 engagement promotes the expression of pro-inflammatory cytokines and type I IFNs.

Several reports show that DCs generated from mobilized monocytes pulsed *in vitro* with TAAs and stimulated with LPS, with or without IFN-γ, were able to express IL-12 and CXCL10, polarize a Th1 immune

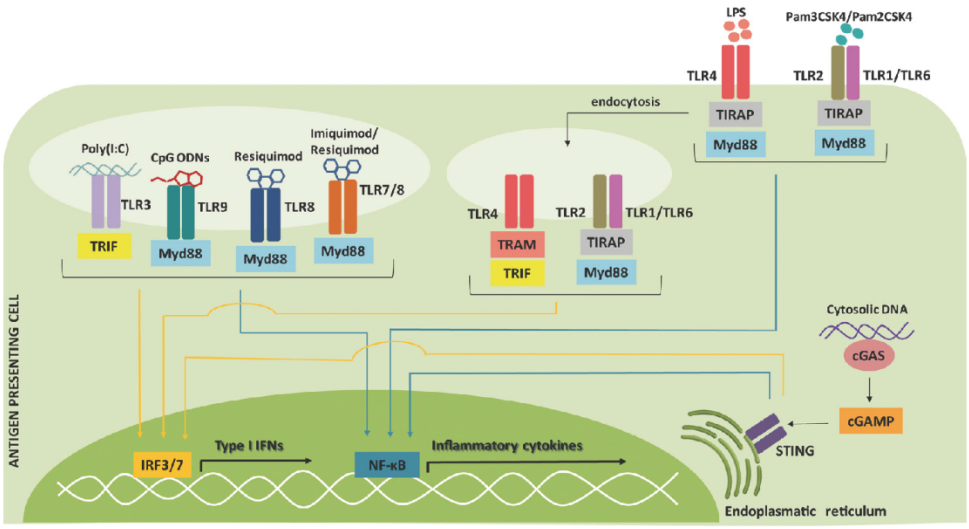

Figure 3: Signaling pathways of type I IFN inducers commonly used as adjuvants for cancer therapy. Nearly all cells are capable of producing type I IFNs after sensing pathogen-associated microbial patterns (PAMPs) and danger-associated molecular patterns (DAMPs). This strategy is used to improve therapeutic cancer vaccines, increasing the immunologic response. The activation of pattern-recognition receptors (PRRs) leads to signaling through the adaptor molecules toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β (TRIF) and/or Myd88, which culminates in the activation of IFN-regulatory factor 3/7 (IRF3/7) (yellow arrows) or nuclear factor-κB (NF-κB) (blue arrows) transcription factors and, consequently, in the expression of type I IFNs or inflammatory cytokines. Regarding the dendritic cell (DC) subtypes, the PRRs toll-like receptor (TLR)1, TLR2, TLR3, TLR4, TLR6 and TLR8 are expressed by monocyte-derived DCs and myeloid DCs, while TLR7 and TLR9 are only expressed by plasmacytoid DC. Lipopolysaccharide (LPS), triacylated lipopeptides (Pam3CSK4), diacylated lipopeptides (Pam2CSK4), polyribosinic-polyribocytidylic acid [Poly(I:C)], oligodeoxynucleotide (CpG ODN), TIR domain-containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM), 2′-3′-cyclic GMP-AMP (cGAMP), cGAMP synthase (cGAS), stimulator of interferon genes (STING).

response and may be useful for DC-based immunotherapy [226–230]. Interestingly, other studies have demonstrated that DCs generated using LPS are capable of inhibiting suppression mediated by $CD4^+CD25^+Foxp3^+$ regulatory T cells [231] and that they restored $CD4^+$ and $CD8^+$ T cell proliferation, while DCs matured with a conventional cocktail (IL-1, IL-6, TNF-α, prostaglandin E2 - PGE2) did not fully restore T cell proliferation [232]. Also, the 3-O-deacylated monophosphoryl lipid A (MPLA) is a less toxic LPS derived from *Salmonella minnesota* R595 and used with alum in a prophylactic vaccine against human papillomavirus 16 and 18 (Cervarix®, GSK Vaccines) [233].

Imidazoquinoline-like molecules

Imiquimod and Resiquimod are imidazoquinoline-like molecules that have been identified as TLR7/8 agonists based on their ability to induce DC maturation. TLR7 is mainly expressed in pDCs and, to some extent, in B cells and monocytes/macrophages [234], while TLR8 is primarily expressed in monocytes/macrophages and myeloid DCs [235]. Therefore, Imiquimod is used specifically to activate pDCs, inducing expression of IFN-α, IL-6, IL-8, IL-12 and TNF-α and stimulating a Th1 immune response; it has been approved by the FDA for treating basal cell skin cancer (Aldara®, 3M Pharmaceuticals) [236].

TLR7 also recognizes single-stranded RNA (ssRNA) derived from RNA viruses (like vesicular stomatitis virus, influenza A virus and human immunodeficiency virus) [237], synthetic poly(U) RNA and certain small interfering RNAs [238], thus pDCs are able to produce large amounts of type I IFN and cytokines in response to virus infection [237]. TLR8 recognizes Resiquimod and viral ssRNA and is upregulated after bacterial infection, having its highest expression in monocytes, although is expressed in several tissues [239].

These sensors utilize the universal adapter protein MyD88, which in turn activates the expression of IRF7 and NF-κB, thereby stimulating transcription of type I and III IFNs, inflammatory cytokines and chemokines [240], especially IFN-α, TNF-α and IL-12 [241]. Upon activation, pDCs also expresses the co-stimulatory molecules CD40, CD80 and CD86 and gain the ability to cross-present antigens in the context of MHC [242].

The use of Imiquimod brought to light intriguing observations regarding DC functions that are not usually considered. Drobits and co-workers showed that Imiquimod treatment promoted the secretion of both TRAIL and granzyme B resulting in pDC-mediated tumor killing [243]. Also, pDCs stimulated with agonists for TLR7 and 9 upregulated the surface expression of TRAIL in a type I IFN-dependent manner, causing the lysis of Jurkat cells and melanoma cell lines SKMel2 and WM793 [244].

The topical treatment of basal cell carcinoma, perianal Bowen's disease and superficial malignant melanomas with Imiquimod led to an increase in activated-pDC infiltration and to a reduction in neoplastic cells with complete regression in some cases [245–248].

Also, Imiquimod may have direct antitumor effects inducing apoptosis via modulation of the expression of Bcl-2/Bax [249–251] and autophagy [252, 253] in several cancer cells, as well as antiangiogenic properties, based on its induction of interferons, IL-10, and IL-12 [254], which end up inhibiting angiogenesis independently of their immunomodulatory functions. In fact, Imiquimod has been successfully used as an antiangiogenic agent to treat vascular proliferative lesions, such as infantile haemangioma, pyogenic granuloma and Kaposi's sarcoma [254–256]. And, in a patient with melanoma, treatment with Imiquimod induced gene expression of angiogenesis or MMP inhibitors, like *IFN-α*, *KiSS1*, TIMP metallopeptidase inhibitor 1 (*TIMP1*), and thrombospondin 1 (*THBS1*), while decreasing expression of *bFGF* and *MMP9*, as shown by quantitative PCR of cutaneous melanoma metastasis biopsies performed before and after treatment [257].

CpG ODNs

The unmethylated CpG ODN (oligodeoxynucleotide) TLR9 agonists are powerful adjuvants for the activation of pDCs. In humans, TLR9 is expressed only in pDCs and B cells [258] and recognizes unmethylated CpG motifs that are found in bacterial and viral genomes [239]. CpG ODNs are divided into four classes depending on the differences in their structure and immunoreactivity. Of these classes, almost all the CpG ODNs used in clinical trials have been class-B CpG ODNs (also known as K-type ODNs), however type I IFN is weakly induced by CpG-B ODNs. Class-A CpG ODNs (also known as D-type ODNs) have also been used but in fewer clinical trials. Class A and C CpG ODNs enter the lysosome compartments of pDCs and B cells to stimulate IFN-α production, while class B CpG ODNs enter the endosomal compartments of pDCs to induce their maturation [259].

CpG ODNs activate TLR9–MyD88–IRF7 and TLR9–MyD88–NF-κB signaling pathways of pDC to induce expression of MHC and costimulatory molecules such as CD40, CD80, and CD86, which results in $CD4^+$ and $CD8^+$ T cell maturation [260] and secretion of type I IFN [261] and IL-6, IL-12 and TNF-α [262]. Additionally, type I IFN and TNF-α secreted from pDCs activate NK and NKT cells [263].

A prospective Phase I trial with stage II–IV metastatic melanoma patients vaccinated with melanoma-associated antigen recognized by T cells-1 (MART-1) peptide, Montadine® ISA-51 (an agonist made of mineral oil and surfactant from mannide monooleate family [264]) and CpG 7909 showed that in the presence of CpG ODN there was 10-fold more MART-1 specific T cells induced in patients [265].

MelQbG10, which is G10 CpG ODN and the tumor peptides MART-1 coated with bacteriophage protein,

was used in combination with Montanide® ISA-51 and topical 5% Imiquimod cream in stage III/IV malignant melanoma patients. Patients vaccinated with MelQbG10 plus Montanide® ISA-51 had significantly higher T cell induction versus MelQbG10 alone, but there were no significant differences in clinical outcome among the different treatment groups [266].

Bacterial triacylated or diacylated lipopeptides

The fatty acid groups of triacylated lipopeptides are recognized by TLR2/TLR1 heterodimers [267], while the fatty acid groups of diacylated lipopeptides are ligands for TLR2/TLR6 heterodimers [268]. Previously, it was thought that TLR2/TLR1 and TLR2/TLR6 engagement elicited the pro-inflammatory pathway, but not the type I IFN responses [269]. It was shown that administration of TLR2 agonists can enhance effector and memory T cell responses, culminating in improved tumor rejection [270, 271]. TLR2 agonists can also increase expression of costimulatory molecules in B cell lymphoma, enhancing its sensitivity to NK and $CD8^+$ T cells [272], or inducing caspase 8-dependent apoptosis [273].

However, recent studies have shown that bacterial ligands can induce type I IFN responses through TLR2 binding. After stimulation, the TLR2 heterodimers are internalized into endolysosomal vesicles, from which they induce IFN-β via MyD88 and IRF1/IRF7 [274] and this pathway requires TRAM [275], but is yet to be fully elucidated. In this way, TRAM acts as adaptor molecule for both TLR4 and TLR2, inducing IRF1 and IRF7 signaling from the endosome.

Dietrich and colleagues observed that stimulation of bone marrow derived macrophages (BMDMs) with Pam3CSK4 (synthetic triacylated lipopeptides TLR2/TLR1 agonist) and Pam2CSK4 (synthetic diacylated lipopeptides TLR2/TLR6 ligand) induced not just pro-inflammatory cytokines like TNF-α and IL-12, but also type I IFN-inducible genes, such as *CXCL-10*, *Mx2*, *IL-6* and *iNOS* [274].

SUP3 is a TLR2 agonist based on the structure of Pam3CSK4, but with a chemically more stable structure. SUP3 was shown to enhance cross-presentation by $CD8^+$ cDCs *in vitro*, up-regulate the expression of CD40 and CD86 co-stimulatory molecules and induce production of IL-6 and TNFα in DC, culminating in an antigen-specific $CD8^+$ T cell response and increased immunization against tumor challenge. SUP3 also induced antigen-specific antibodies such as IgM, total IgG and high affinity IgG [276].

2′-3′-Cyclic GMP-AMP

Several authors have shown that 2′-3′-cyclic GMP-AMP (cGAMP) can be used directly as an adjuvant for antitumor therapy [277]. cGAS is a major sensor of cytosolic DNA, irrespective of the DNA sequence [278, 279]. Cytosolic DNA can trigger strong production of type I IFNs and other inflammatory cytokines in immune and non-immune cells. After DNA binding, cGAS undergoes a conformational change that promotes the conversion of GTP and ATP into cGAMP [280]. cGAMP then acts as a second messenger that activates the adaptor protein STING, at the endoplasmic reticulum membrane [279]. STING in turn activates the proteins inhibitor of nuclear factor-κB kinase (IKK) and TANK binding kinase 1 (TBK1), which activate NF-κB and IRF3, respectively, inducing production of cytokines and type I IFN [281].

DCs can also activate the cGAS–STING pathway after DCs phagocytose tumor cells and some of the tumor DNA escapes to the cytoplasm. Woo and co-workers (2014) showed that mice deficient for Myd88, TRIF, the purinergic receptor P2X7 (P2XR7), mitochondrial antiviral-signaling protein (MAVS) or retinoic acid inducible gene I (RIG) had no defect in priming of $CD8^+$ T cells. Strikingly, in both STING-deficient and IRF3-deficient mice, there was a substantially diminished $CD8^+$ T cell response against tumor-associated antigens and, in wild-type mice, transfer of tumor DNA to host APCs resulted in TBK1 and IRF3 phosphorylation and, as a consequence, production of IFN-β [282]. ECs are also producers of type I IFN in response to STING activation. Demaria and collaborators showed that intratumoral injection of exogenous cGAMP enhanced STING activation in the tumor microenvironment, resulting in stimulation of type I IFN response and antitumor $CD8^+$ T cells, leading to growth inhibition of injected and contralateral tumors. Interestingly, this effect resulted mainly from tumor ECs, which were the main producers of IFN-β in response to cGAMP injection in both mouse and human [283].

In a study performed by Wang and co-authors, PD-L1 antibody was administered in a mouse model of melanoma and they observed that cGAS-deficient mice are refractory to the antitumor effects of a PD-L1 antibody. They showed a large increase of tumor-infiltrating leukocytes in wild-type mice after PD-L1 antibody treatment, but not in cGAS- or STING-deficient mice. This may be due to tumor cell killing caused by PD-L1 antibody treatment, which exposes tumor-associated antigens and DNA that are taken up by DCs. Then, tumor DNA escapes to the cytoplasm of DCs and activates the cGAS–STING pathway, inducing the production of type I IFN and the co-stimulatory molecule CD86, and activating a Th1 response. When they applied cGAMP intramuscularly, this caused inhibition of tumor growth and prolonged mouse survival after PD-L1 antibody treatment [284].

Previous studies have shown that intratumor injection of cGAMP and its analogs also induced antitumor effects. However, some authors suggest that STING activation may induce a suppressive tumor microenvironment and contribute to tumor growth and metastasis [285]. Metastatic brain cancer cells generate cGAMP, which is transferred by gap junctions to astrocytes, activating the STING pathway in these cells and producing proinflamatory cytokines, which in turn activate STAT1 and NF-κB pathways in the metastatic cells, thus supporting tumor growth [286].

Immunogenic cell death

During the last decade, the newly defined concept of immunogenic cell death (ICD) induced a thorough revision to the previously accepted, classic point of view cell death as a dichotomized phenomenon as either apoptotic, associated with a tolerogenic immune response that maintains tissue homeostasis or, in contrast, necrotic, a promoter of the inflammatory response [287]. Undeniably, along with the success of checkpoint blockade immunotherapy [288], ICD helped to cement the importance of the immune system during cancer treatment, especially pertinent when selecting which chemotherapy to administer since unsuccessful approaches are often immunosuppressive and unable to activate antitumor immunity.

ICD was originally demonstrated as a cellular and molecular response of cancer cells to anthracyclines that involves the exposure and secretion of immunogenic DAMPS, in a defined temporal sequence, providing both antigenic and stimulatory signals for the DC compartment to generate an effective $CD8^+$ T cell attack against remaining tumor cells [287]. The first study that unveiled this mechanism showed that *ex vivo* treatment of MCA205 sarcoma cells with doxorubicin and subsequent inoculation of these dying tumor cells into naïve syngeneic mice protected them against a subsequent tumor challenge. Remarkably, this protection was not seen when cells were treated with mitomycin C (another chemotherapeutic agent) or when caspase-3 activity was blocked, showing that a specific property of cell death induced by doxorubicin was mediating immune stimulation. Furthermore, intratumoral application of doxorubicin in subcutaneous established tumors only exhibited therapeutic efficacy when treatment was performed in immunocompetent mice, whereas treatment in the nude background (lacking mature T cells) or in animals depleted of DCs abolished the immune response against the tumor cells [289].

Key mechanistic insights came later when Obeid and colleagues used a large panel of apoptosis inducers and identified changes in the plasma membrane proteome that were exclusively present in anthracycline treated cells and not in the presence of the pan-caspase inhibitor. Comparison of two-dimensional electrophoresis, followed by mass spectroscopic analyses, led to the identification of the endoplasmic reticulum chaperone calreticulin (CRT). Accordingly, knockdown of CRT negatively affected phagocytic uptake of dying tumor cells by DCs and abrogated the immune protection effect. Therefore, CRT release (ecto-CRT) was the first key feature identified as a determinant of the interaction of DCs and dying immunogenic cells and consequently the anticancer immune response [290].

Furthermore, as revealed in subsequent studies, other ICD determinants have been identified. This way, the proposed mechanism postulates that: (i) in response to lethal insult from doxorubicin treatment, dying tumor cells activate autophagy machinery and secrete ATP that, in turn, is recognized by purinergic receptors (P2RY2 and P2RX7) of DCs, promoting DC recruitment and activation; (ii) exposition of Annexin A1 mediates DC contact with the dying tumor cell; (iii) secretion of CRT, as a consequence of endoplasmic reticulum stress, acts on antigen uptake by DCs; (iv) release of the alarmin high-mobility group box 1 (HMGB1) from the nucleolus, which then binds to TLR4 of DCs to induce full maturation status, secretion of IL-1β and eventually leading to priming of T cells with complete cytotoxic capacity [291].

Interestingly, all of these processes regarding release and secretion of ICD markers alone or in combination cannot predict with certainty if the cell death process will truly be immunogenic, which suggests that additional, unknown factors remain to be identified [292]. Along these same lines, recently a novel mechanism was uncovered: the cancer cell–autonomous secretion of type I IFNs [293].

The role of type I IFNs in ICD was revealed by analyzing immunologically relevant transcriptional changes induced in sarcomas upon intratumoral treatment with doxorubicin. Among the modulated pathways, transcripts associated with response to viral infections were indicated by type I IFN stimulated-genes, pointing to a type I IFN fingerprint in the cancer cells. Indeed, antibody mediated blockade of IFNAR1 or IFN-α/β neutralization markedly inhibited doxorubicin's antitumor effects. Sarcoma tumors derived from an *Ifnar2*$^{-/-}$ background did not respond to doxorubicin when transplanted into a wild-type host, suggesting that the IFN-β produced during ICD was most likely affecting the tumor cells, not the host immune system. As further demonstrated, *Ifnar1*$^{-/-}$ tumor cells failed to secrete CXCL10 in response to doxorubicin, just as was also seen in tumor cells derived from *Tlr3* KO or its adaptor *Trif*. These data demonstrated that ICD inducers act by stimulating IFN-β secretion through an autocrine and paracrine mechanism that takes place upon TLR3 recognition of self RNA from dying cells, activating the CXCL10-CXCR3 signaling axis to attract effector immune cells [293].

In accordance with these findings, radiation therapy (RT), a known ICD inducer, was also reported to depend on IFN-β signaling. In the work of Burnette and collaborators, local ablative RT of B16-SIY tumors resulted in striking tumor regression and local production of IFN-β by tumor-infiltrating $CD45^+$ cells. In agreement, tumor associated DCs presented higher levels of maturation markers (CD40, CD80 and MHC-I and II molecules) after RT, yet if treatment was performed in an *Ifnar1* KO background, therapeutic control was completely lost. Additionally, to determinate the host compartment in which IFN-β was necessary, bone marrow transplants from *Ifnar1* KO mice to a WT host revealed a requirement for the hematopoietic cells, more specifically the $CD11c^+$ and $CD11c^-$ myeloid populations, that in a context lacking IFN-β lost their cross-priming capacity within the tumor microenvironment [294].

Another ICD inducer that relies of type I IFN activity is the oncolytic Newcastle Disease Virus (NDV) [295], which is an anticancer virotherapy strategy, and as such, was expected to be impaired by the antiviral properties of IFNs, thus negatively affecting therapy outcome. However, as demonstrated in the work of Zamarin and colleagues, intratumoral applications of the oncolytic NDV accompanied with CTLA-4 checkpoint blockade promoted complete regression of B16 tumors in both primary and non-treated secondary sites, showing a remarkable systemic immune protection. Additionally, the authors found that this immune protection was mediated by both NK and $CD8^+$ T cells, and, unexpectedly, if treatment was performed in a *Ifnar1*$^{-/-}$ host, therapeutic efficacy was abrogated in the injected tumors as well as in the contralateral challenge, even when combined NDV plus CTLA-4 treatment was applied [296].

Three pathways of innate immune sensing can lead to *Ifnb* gene transcription: (i) TLR stimulation signals through MyD88 and TRIF adaptors, (ii) RIG-I senses cytosolic RNA and signals through the adaptor protein IPS-1, and (iii) STING senses cytosolic DNA and promotes type I IFN expression [297, 298]. Consequently, induction of type I IFN in the tumor microenvironment correlates with T cell infiltration. Gajewski demonstrated, through melanoma gene expression profiling, that tumors infiltrated with $CD8^+$ T cells also exhibited a type I IFN transcriptional signature [299], a suggestion that type I IFN signaling might participate in innate recognition of tumors [157]. Also, as said before, mice deficient in type I IFN response showed decreased spontaneous T cell priming in transplantable tumor models and increased tumor induction using methylcholanthrene [6].

Overall, the findings presented here highlight the critical role of integrated innate and adaptive immune responses in order to achieve full therapeutic efficacy and, most importantly, revealing type I IFN signaling as an indispensable propeller of the cancer immunity cycle. Yet, it remains to be determined which tumors are likely to benefit from ICD treatment, for example, one report indicates that spontaneous mammary tumors in (MMTV)-NeuT transgenic female mice can successfully respond to immunogenic chemotherapy even in the absence of the immune system [300]. But, as shown by Pfirschke and collaborators, pretreatment with the combination of oxaliplatin-cyclophosphamide can increase T cell infiltration in resistant *Kras/Trp53* mutant tumors, rendering them sensitive to anti-PD-1 and CTLA-4 blockade [301], suggesting the potential benefit of associating chemo and immunotherapy. However, inducing ICD using multiple inflammatory immunotherapeutic agents may not boost immune attack, since negative regulatory mechanisms will likely be stimulated and, thus, impede the immune response.

TYPE I IFNS IN CANCER GENE THERAPY: TARGETING TUMOR AND DENDRITIC CELLS

According to the Journal of Gene Medicine, 2409 clinical trials making use of gene transfer approaches have been included in their online database since 1989, of which 1554 (approximately 64.5% of the total) were aimed at treating cancer. Cancer gene therapy is expected to remediate faulty gene function in order to kill tumor cells or render them susceptible to killing by chemo/radiotherapy or immune attack. Such approaches may include the silencing of oncogenes [302], the transfer of tumor suppressor genes, generally aiming to trigger mechanisms of cell death [303], and the transfer of immune modulating genes in order to elicit antitumor systemic responses [304, 305]. As will be discussed further, the gene therapy approach may not target the tumor cell directly, but can be used to trigger an anti-tumor immune response, such as in the case of vaccines based on modified DCs.

Many vectors have been employed for the transfer of type I IFN genes, including for modification of tumor cells or DCs (Table 1). Among these vectors, adenovirus, AAV and, more recently, non-viral liposome mediated gene transfer have been used in both basic and clinical research protocols [306–311]. Specifically in the case of IFN-α gene transfer, adenovirus is one of the most commonly employed vectors for *in situ* treatment models, yet lentivirus is better suited for *ex vivo* creation of IFN-α secreting cells.

In preclinical models, as expected from its antitumor functions, IFN-α gene transfer induced: (i) cell cycle arrest, (ii) apoptotic cell death mechanisms [312–314], (iii) decreased hemoglobin index and microvessel density, and (iv) necrotic ischemia in tumor tissue [312, 313, 315]. Regarding the immunomodulatory property of IFN-α, it was also observed that the gene transfer, alone or combined with other therapy approaches, lead to: (i) increased presence of infiltrating $CD8^+$ and $CD4^+$ T cells and decreased $Foxp3^+$ cells in the tumor parenchyma and also augmented MHC-I expression on tumor cells [316]; (ii) enhancement of NK cell cytolytic activity [312] and (iii) increased presence of $CD11c^+$ cells in regional lymph nodes [317]. In this way, the immunosuppressive features of the tumor microenvironment can be overcome upon IFN-α gene transfer and indications of a systemic antitumor immune response are uncovered.

More recently, the effect of the IFN-α on metastasis was investigated. In this approach, hematopoietic stem cells (HSCs) were modified with a lentiviral vector in order to generate $Tie2^+$ macrophages/monocytes that constitutively express IFN-α. The natural homing of these cells to tumor sites was observed, leading to a reduction in hepatic metastases from colorectal cancer, without exerting a negative influence on the homeostasis of hematopoiesis [318, 319].

Table 1: Properties of the main vectors used in gene therapy protocols

Vector	Integrative	*in vitro* delivery efficiency	*in vivo* delivery Efficiency	Capacity to trigger immune response	Efficient production
Adenoviral	No	High	High	High	Yes
Lentiviral	Yes	High	High	Low	No
Retroviral	Yes	High	High	Low	No
AAV	No	High	High	High	No
Liposomes	No	High	Low	Low	Yes

Abbreviations: AAV, Adeno-associated virus.

In light of the successful results obtained from IFN-α gene transfer to cancer in basic-research models, a phase-I clinical trial was carried out using a recombinant adenoviral vector to treat non-muscle invasive bladder cancer in which 17 patients were enrolled. The subjects received escalating doses of the vector and the gene transfer efficacy was assessed by examining cytokine levels in the urine. Even at the highest dose, the treatment was well tolerated, with only mild adverse events being observed. Regarding the treatment efficacy, it was reported that 7 patients achieved complete response at 3 months [320].

In a similar way, in basic research protocols aiming to assess the effects of IFN-β gene transfer in both *in vitro* and *in vivo* models, it was observed that the treatment: (i) reduced tumor cell proliferation [133, 321–323]; (ii) decreased cell viability in both monolayer and spheroids cultures [308, 322]; (iii) increased long-term survival with reduced tumor burden [311, 323–327] and (iv) reduced tumor volume without notable toxicity, yet an increase in apoptotic cells and areas of necrosis in tumor tissue [133, 321, 324]. In this way, IFN-β gene transfer may offer an advantage, localized high concentrations of this protein, which cannot be achieved with biochemotherapy.

Delving into the effect of gene transfer on the microenvironment and immune system, in preclinical models after treatment it was reported that: (i) the cells presented downregulation of genes associated with angiogenesis, such as *bFGF*, *MMP9*, *VEGF-A* and *IL-8* [311, 322]; (ii) there was decreased quantity and density of blood vessels and diminished levels of hemoglobin in the tumor [311, 327]; (iii) treated animals were less prone to develop spontaneous metastasis, became resistant to a second tumor challenge or to the establishment of induced metastases [323, 326, 327]; (iv) increased infiltrating $CD8^+$ T lymphocytes [323, 326, 328], activated NK cells [326, 329] and macrophages [133, 324, 329] as well as increased levels of MHC-I on the tumor cells [326]. The efficacy of IFN-β gene therapy was considerably decreased only in animals depleted of $CD8^+$ T cells, indicating that this class of lymphocytes play a critical role in the immunomodulation stimulated by IFN-β [328].

Although many vectors are being used for gene transfer, armed oncolytics carrying IFN-β has recently gained ground. Approaches using the Vesicular Stomatitis Virus (VSV) encoding IFN-β have been shown to elicit a strong antitumor immune response, decreasing infiltrating T-reg cells and increasing $CD8^+$ cells, and also stimulating the expression of PD-L1 on tumor cells [330]. Since VSV-IFN-β offers increased capacity to elicit both innate and adaptive immune responses as well as preferential replication in tumor cells, it is safer and more effective as compared to VSV with no transgene [331, 332], features that led to the establishment of a phase I clinical trial in 2012, that is still ongoing with estimated primary completion date in June 2017 (https://clinicaltrials.gov/ct2/show/study/NCT01628640).

Despite the positive responses seen in preclinical studies, only a few clinical protocols using recombinant vectors for the delivery of IFN-β in cancer patients have been carried out [333–335]. As an example, in a phase I clinical trial using a recombinant adenoviral vector, 11 patients with recurrent malignant glioma received different doses of the vector by stereotactic injection in the tumor site. The first injection was performed approximately one week before the scheduled tumor resection surgery and a second right after the procedure. After the treatment regimen it was clear that the vector treatment reproduced some data from the preclinical models, i.e, induction of apoptosis and the presence of necrotic areas in the treated tissue. Although a treatment-related dose limiting toxicity was seen in one patient enrolled in the highest dose cohort, IFN-β gene therapy was shown to be a safe and potentially effective approach [336]. On the other hand, despite its proven safety, it was also seen in another clinical trial that repeated doses of the adenoviral vector did not considerably improve the clinical outcome in patients with mesothelioma due to the fast development of neutralizing antibodies against the vector, an obstacle that, perhaps, could be circumvented by using a non-immunogenic vector or by combining gene transfer with additional therapeutic approaches [332].

Modified dendritic cells expressing type I interferons: crossroad between cancer vaccines and gene therapy

In order to induce a host immune response against tumor cells, genetically modified DCs have been used in vaccination protocols. In spite of the clear rationale, DC-based vaccination faces some technical obstacles, such as

the best condition for their activation such that the antitumor immune response is efficiently induced [337]. Given the influence of type I IFNs on DCs, it is reasonable that IFN-α/β gene transfer be used to activate DCs and positive results have been reported. For example, mice bearing GL261 glioma tumors were treated with one intratumoral injection of an adenoviral vector encoding IFN-α followed by implantation of syngeneic bone marrow-derived DCs resulting in increased survival due to an antitumor immune response dependent on $CD8^+$ T cells, and it was shown that the animals acquired a certain level of resistance against a second tumor challenge [338]. In another model the vaccination protocol consisted of intratumoral delivery of DCs previously modified with a recombinant adenoviral vector encoding IFN-α in combination with irradiated tumor cells engineered to express IL-4 or GM-CSF, resulting in stronger tumor specific CTL responses in the cervical lymph node and increased survival [339].

Specifically regarding the genetic modification of DCs, many recombinant vectors have been used, such as those derived from adenovirus, lentivirus, retrovirus, AAV and Sendai virus [340]. In spite of the many types of vectors available for the genetic modification of DCs, adenoviral vectors are most commonly used since they provide highly efficient gene transfer and expression, easy handling and high-titer preparations. Even so, adenoviral vectors present some disadvantages, e.g. pre-existing neutralizing antibodies and transductional dependency on the coxsackievirus and adenovirus receptor (CAR) [341]. However, the former should not present a barrier when virus is applied to DCs *ex vivo* and the latter can be overcome with the use of modified adenoviral vectors that no longer depend on CAR. Still, adenoviral vectors trigger molecular mechanisms leading to the maturation of DCs and, as a consequence, a more consistent antitumor immune response [342, 343].

In addition to adenovirus, other vectors have been gaining space in the field of genetically modified DCs. For example, non-viral methods are being more frequently used, including mRNA transfection, due to their lower manufacturing costs, comparable levels of expression, and relative transfection efficacy, especially with the use of electroporation [344]. Another emerging viral vector is the Sendai virus due to its high transduction efficiency *ex vivo* and *in vivo*, augmented capacity to trigger antitumor immune responses and elicit DC maturation [345, 346]. Along the same lines, exploring the properties of IFN-α produced by pDCs, infection of pDCs with a replication-deficient herpes simplex virus 1 (HSV-1) *d*106S vaccine strain showed a robust cytotoxic effect against various melanoma cell lines that was equivalent or superior to the effects induced by synthetic TLR7 and TLR9 agonists [347].

p19Arf and interferon-β combined gene transfer

As presented above, gene transfer of IFN-α/β directly into the tumor mass or into dendritic cells presents promising results that merit further development for clinical application. However, if one aims to increase the intrinsic antitumor and immunomodulatory properties of type I IFNs, which strategy should be used? More specifically, using a gene transfer method, how can we assure that most of the transduced tumors cells would die? Since type I IFNs are already considered sufficiently immunogenic, can we enhance their immune stimulation?

In light of these questions, our lab has previously developed a set of unique adenoviral vectors which utilize a p53 responsive promoter, called PGTxβ [348] to direct the expression of the cDNAs for $p19^{Arf}$ ($p19^{Arf}$ for mouse and $p14^{ARF}$ for humans) and for IFN-β. Arf is a tumor suppressor protein that is encoded by the CDKN2A locus (also encoding the $p16^{INK4a}$ protein) [349] and is mainly known for being a functional partner of p53, since after oncogenic stress Arf associates with MDM2 and prevents MDM2 mediated ubiquitination of p53 for posterior degradation [350]. Thus, Arf can enable p53 to trigger growth arrest, apoptosis and also acts in a p53 independent manner by inhibiting ribosomal RNA processing, promoting apoptosis and regulating autophagy [351, 352].

By combining Arf and IFN-β along with the p53 responsive promoter, we hoped to create interplay between: (i) transgene control, (ii) p53/Arf pro-apoptotic functions and (iii) IFN-β antiviral and immunomodulatory activities. Indeed, other studies have already pointed potential benefits of targeting the p53/Arf/IFN-β pathways, but never explored its therapeutic application. For example, Takaoka and collaborators have shown that IFN-α/β activates p53 transcription and stabilizes its protein levels [353]. Interestingly, they showed that p53 and type I IFNs work cooperatively to potentiate the apoptotic machinery and mediate tumor suppression and viral control functions. Furthermore, Sandoval and colleagues showed that apoptosis induced by type I IFNs requires p14Arf, but not p53, since human sarcoma cells null for $p14^{ARF}$ undergo apoptosis when $p14^{ARF}$ is reintroduced in the presence of IFN-α/β, but the same observation is not seen with p53 [354].

Based on this evidence, we decided to explore the murine B16F10 (B16) melanoma cell line as a model since it harbors p53 in its wild type form, as seen in 90% of human melanoma cases [355] and is a well-known model for immunotherapies. Remarkably, in our initial observations we noticed that combined gene transfer of $p19^{Arf}$ and IFN-β, but not the either single treatment, provoked massive cell death while up-regulating p53 target genes $p21^{Waf1}$, *Mdm2* and *Puma* [356].

Evidence for superior immune stimulation came from two distinct immunization contexts. In the first, mice were vaccinated prophylactically with *ex vivo* transduced B16 cells that while dying were inoculated in naïve syngeneic C57BL/6 mice and seven days later, mice were challenged with fresh B16 cells in the contralateral flank. Tumor formation was completely abrogated at the vaccine site in hosts with competent NK cell compartment due to

the up regulation of *Il-15*, *Ulbp1* NK cell receptor, *Killer/Dr5* and *Fas/Apo-1* death receptors on the treated cell, thus providing a safety benefit for the combination. At the challenge site, a dramatic decrease in tumor progression was observed and was dependent on tumor-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes. Unexpectedly, in this prophylactic model, IFN-β alone or in combination with $p19^{Arf}$ showed similar protection and T cells presented similar killing capabilities and levels of IFN-γ and TNF-α secretion. This would argue that there was no evident immunological superiority for the combination. However, when exploring a therapeutic tumor model, where the tumor challenge was made before the immunization step, only $p19^{Arf}$/IFN-β vaccinated mice displayed reduction in tumor progression [357].

In support of this evidence, now in the second immunization context, mice bearing heterotopic (s.c) lewis lung carcinoma (LLC1) tumors were treated with four rounds of adenoviral injections directly into the tumor mass and subsequently challenged with fresh LLC1 cells in the opposite flank. Remarkably, mice that had their primary tumor treated with the $p19^{Arf}$/IFN-β combination showed improved tumor control at the challenge site even when compared to IFN-β single treatment, showing superior immune protection by the combined $p19^{Arf}$ and IFN-β *in situ* gene therapy [358].

Furthermore, seeking to gain mechanistic insights on how the combination could induce cell death and immune stimulation, we evaluated the transcriptional profile of critical protein pathways, revealing that only the $p19^{Arf}$/IFN-β combination induced genes related to both the p53 pathway and apoptosis as well as IFN-β immune response and antiviral functions [359]. We also noted that the use of the adenoviral vector was a critical component for inducing cell death, reinforcing the antiviral aspect of the response. Intriguingly, the $p19^{Arf}$/IFN-β combination promoted cell death by a different mechanism than that seen for the individual treatments, since inhibition of capase-3/7 increased the levels of cell death upon the individual treatment with $p19^{Arf}$ or IFN-β, but did not affect the $p19^{Arf}$/IFN-β group, suggesting that a caspase-independent mechanism of cell death was induced by the combined treatment. Of the three groups, the combination showed the lowest caspase 3 activity, while displaying features of necroptotic death, as revealed by the increase of Rip-3 (key mediator of necroptosis) and TNF receptor (*Tnfrsf1A*, an activator of the necrosome complex). Moreover, consistent with the recent demonstration that necroptosis can promote ICD [360], only the combined gene transfer of $p19^{Arf}$ and IFN-β was accompanied by the exposition of calreticulin, ATP secretion and HMGB1 release, providing mechanistic support for the immunomodulatory superiority of the combination [359].

Taken together, we believe that our data provide functional and mechanistic evidence to classify our $p19^{Arf}$ and IFN-β combined gene transfer as a novel agent for cancer immunotherapy. In fact, to the best of knowledge, no other gene transfer strategy employing non-replicating viral vectors has been shown to unleash ICD. Although we have identified NK cells, $CD4^+$ and $CD8^+$ T lymphocytes as critical cell mediators, we do not fully understand the mechanism by which these cells cooperate to bring about the anititumor immune response, most importantly we have not yet analyzed how DCs are being affected. Since NK cells can assume a helper phenotype to modulate DC priming function [178], it will be interesting to investigate how the NK cells activated in the vaccine site are interacting with DCs and promoting antigen uptake in our approach. Along the same lines, we expect that the $p19^{Arf}$/IFN-β combination will provide not only an IFN-β immunomodulatory stimulus, but also immunogenic DAMPs unleashed during the ICD process, which together may provide an ideal stimulus for DC maturation, especially in the immunosuppressive tumor microenvironment. Another possibility regarding DCs would be to generate *ex vivo* derived DCs and use treated dying tumor cells as adjuvant as well as a source of antigen, an application that was successfully demonstrated in a pre-clinical model of high-grade glioma tumors [361]. Nevertheless, despite the long road ahead, IFN-β gene transfer and its combination with Arf holds a promising position in the cancer immunotherapy field.

CONCLUSIONS

Type I IFNs certainly play a critical role in the anti-cancer immune response and represent attractive strategy for therapy. Indeed, considering immunomodulation of the tumor microenvironment and its components, including stroma, immune and tumor cells, type I IFNs can be exploited by several strategies, such as inducers of ICD, agonists of TLRs, gene therapy and recombinant protein for the treatment of cancer (summarized in Figure 4). However, as discussed above, owing to their complex regulatory mechanisms, depending on the model of study, therapeutically induced type I IFNs have been shown to be required for either tumor cells or for infiltrating immune cells, especially DCs, to affect an anti-tumor response. Studies that can obtain deeper mechanistic insights are surely needed to clarify this dual requirement. For example, using a therapeutic model of systemic poly A:U application, Nocera and colleagues have visualized IFN-β in the tumor microenvironment, identifying the $CD11c^+$ population as the main host source of IFN-β, but not the only one [362]. In this model, host type I IFN signaling was absolutely required for therapeutic efficacy and for poly A:U induced antitumor immunity. Moreover, using the same IFN-β luciferase reporter mouse, Lienenklaus and collaborators have previously revealed tissue-specific expression of IFN-β following infection with influenza or La Crosse virus, but most importantly, that IFN-β is constitutively expressed in low amounts by several tissues,

including thymic epithelial cells, to maintain an activated state prepared for infection by pathogens [363].

Nevertheless, having in mind the central position of DCs in the cancer immunity cycle and the profound immunomodulation exerted by type I IFNs, even in the scenario that type IFNs are mainly impacting tumor cells, it would be reasonable to expect that antigen presenting functions of DCs are also being affected. Moreover, the dynamics and expression levels are also important factors to be considered, for example, should a tumor that already displays an ISG signature or a $CD8^+$ T cell infiltrate be treated with the same amount of type I IFNs as compared to tumors that do not? Along the same lines, taking in to consideration the adaptive resistance mechanisms observed both in tumor and host cells, would an IFN based treatment in an inflamed tumor simply favor immunosuppression [184]? Some of the other major hurdles that must be overcome include the toxicity seen when high-dose recombinant protein is administered systemically as well as the relatively bland response encountered when IFN-α/β are applied as single agent gene therapies. We and others propose that more sophisticated ways to deliver a more localized concentration of type I IFN along with a tighter control over expression dynamics would alleviate adverse effects while still providing the desired biological effect. Gene therapy continues to be a promising method for the delivery of IFN-α/β in such manner, though we have strong evidence that the delivery

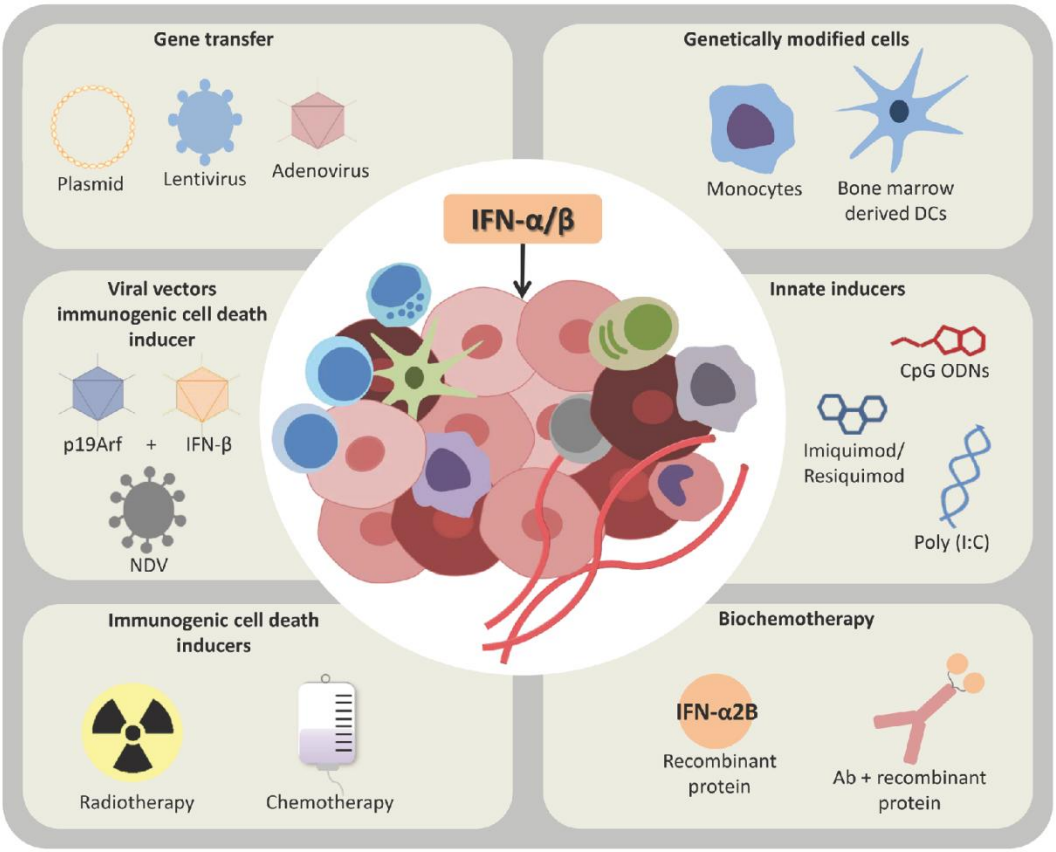

Figure 4: Harnessing type I interferons in cancer therapy. During the last decades several strategies have been developed in order to exploit the antitumor properties of type I interferons (IFNs) in the tumor microenvironment. Indeed, diverse strategies range from the stimulation of tumor cells to produce their own IFN-α/β [e.g., inducers of immunogenic cell death, agonists of toll like receptors (TLRs) and gene therapy] or to deliver it to the cancer microenvironment, for example recombinant protein or dendritic cells (DCs) modified *ex vivo*. Though there is no consensus on which strategy is likely to provide the best results and much more remains to be understood concerning type I IFN's pleiotropic functions, its combination with other treatment modalities, such as checkpoint blockade immunotherapy, is expected to unleash the full force of the immune system against cancer. Newcastle disease virus (NDV), antibody (Ab), oligodeoxynucleotide (CpG ODNs), polyribosinic-polyribocytidylic acid [Poly(I:C)].

of a second factor may be critical to releasing the full force of ICD. Even so, more experimentation is necessary to identify novel partners for IFN-α/β, including their pairing with chemo/radiotherapy and checkpoint blockade. Key regulators of the interplay between IFN-α/β, DCs and immune activation are still being revealed and, we propose, will continue to play an ever more critical role in cancer therapy.

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CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest regarding the publication of this article.

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Vaccination using melanoma cells treated with p19Arf and interferon-beta gene transfer in a mouse model: a novel combination for cancer immunotherapy

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Supplemental information avaliable at Appendix B

Vaccination Using Melanoma Cells Treated With P19Arf and Interferon-Beta Gene Transfer: A Novel Combination for Cancer Immunotherapy

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Keywords: cell death, p19Arf, interferon-beta, immunotherapy, melanoma, adenovirus

Précis: Melanoma cells dying due to combined p19Arf and interferon-beta gene transfer used as a novel immunotherapy promote NK cell activation and increased survival in a prophylactic vaccine model and decreased tumor progression in a therapeutic model.

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Abstract

Previously, we combined $p19^{Arf}$ (*Cdkn2a*, tumor suppressor protein) and interferon-beta (IFNβ, immunomodulatory cytokine) gene transfer in order to enhance cell death in a murine model of melanoma. Here, we present evidence of the immune response induced when B16 cells succumbing to death due to treatment with $p19^{Arf}$ and IFNβ are applied in vaccine models. Use of dying cells for prophylactic vaccination was investigated, identifying conditions for tumor-free survival. After combined $p19^{Arf}$ and IFNβ treatment, we observed immune rejection at the vaccine site in immune competent and nude mice with normal NK activity, but not in Nod-scid and dexamethasone immunosuppressed mice (NK-deficient). Combined treatment induced IL-15, ULBP1, FAS/APO1 and KILLER/DR5 expression, providing a mechanism for NK activation. Prophylactic vaccination protected against tumor challenge, where markedly delayed progression and leucocyte infiltration were observed. Analysis of primed lymphocytes revealed secretion of TH1 related cytokines and depletion protocols showed that both $CD4^+$ and $CD8^+$ T lymphocytes are necessary for immune protection. However, application of this prophylactic vaccine where cells were treated either with IFNβ alone or combined with $p19^{Arf}$ conferred similar immune protection and cytokine activation, yet only the combination was associated with increased overall survival. In a therapeutic vaccine protocol, only the combination was associated with reduced tumor progression. Our results indicate that by harnessing cell death in an immunogenic context our $p19^{Arf}$ and IFNβ combination offers a clear advantage when both genes are included in the vaccine and warrants further development as a novel immunotherapy for melanoma.

Introduction

One key aspect of melanoma, in comparison with other cancers, is that p53 remains wild-type in 80-95% of the cases and this may represent an interesting therapeutic opportunity (1). Under homeostasis conditions, p53 (TP53) levels are maintained low mainly because of its inhibitors MDM2 and MDMD4 that target p53 for proteasome mediated degradation (2). However, under oncogenic stress the protein $p19^{Arf}$ ($p19^{Arf}$ for mice and $p14^{ARF}$ for humans) binds directly to MDM2 or re-locates it to the nucleolus, blocking the MDM2 mediated degradation of p53. Anti-tumor functions of Arf are not fully elucidated, though it can act in a p53-independent manner to regulate ribosomal biogenesis, transcription, response to DNA damage, apoptosis and autophagy (3). Deletions in the $p19^{Arf}$ gene (*Cdkn2A*) occur in wild-type p53 expressing melanomas that do not have MDM2 and MDM4 amplifications, suggesting that inactivation of p53 function can be established by either overexpression of MDM2 and MDM4 or loss of Arf (1).

Among the current immunotherapies recommended for the clinical management of patients with high-risk and advanced-stage melanoma, interferon-alpha2 (IFNα2) has been indicated as an adjuvant therapy (4). As a type I interferon, most effects of IFNα and IFNβ are believed to be immunomodulatory, causing an up-regulation of STAT-1 and an infiltration of dendritic cells (DC) and T lymphocytes into the tumor bed (5). Moreover, it has been reported that IFNs can modulate more than 300 genes, mainly through the JAK-STAT pathway to exert apoptotic, antiangiogenic and immunomodulatory effects that are critical for immune rejection of the tumor (6, 7).

Previously, our group has developed a synthetic p53-responsive promoter, called PGTxβ, and used it to drive transgene expression in an adenoviral vector (AdPG) (8). AdPG was then used to successfully mediate gene transfer of $p19^{Arf}$ or IFNβ cDNAs to the B16 mouse melanoma cell line (Arf deficient and p53 wild-type) in an endeavor to associate cell death and antitumor immunity. Strikingly, we observed that, *in vitro*, cell death was significantly enhanced by the combined gene transfer ($p19^{Arf}$ plus IFNβ) when compared to the single therapies and also showed correlation with the up-regulation of transcriptional targets of p53. Furthermore, in a mouse model of *in situ* gene therapy, treatment of subcutaneous tumors by $p19^{Arf}$ and IFNβ combination decreased tumor progression and increased cell death *in situ*, thus prolonging survival (9). Here we aim to investigate the anti-tumor immune response elicited by the combined $p19^{Arf}$ and IFNβ treatment in mouse models of an anti-melanoma vaccine.

Materials and Methods

Cell culture

The mouse melanoma cell line B16mCAR (hereafter called B16) was originated by the modification of the B16F10 cell line with forced expression of the murine Coxsackie Adenovirus Receptor as previously described (9). The TM1 mouse melanoma cell line, kindly provided by Dr. Roger Chammas (FMUSP), was maintained as described (10).

Vector construction, virus production and detection of transgene expression

Construction of the p53-responsive adenoviral vectors (AdPG, non-replicating serotype 5) encoding the mouse cDNAs for $p19^{Arf}$ or IFNβ as well as virus production have been described previously (9). Titration of adenoviral stocks was performed with the Adeno-X Rapid Titer Kit (Clontech) where titer yields were: AdPGLUC ($2.8x10^{11}$ IU/mL (infectious units/milliliter), AdPGp19 (10^{11} IU/mL) and AdPGIFNβ ($3.2x10^{11}$ IU/mL). Detection of $p19^{Arf}$ and IFNβ was performed as described in Merkel et al (9).

Animal studies

C57Bl/6 (7 week old, female) and NUDE (*Foxn1*^{n}, 7 week old, female) mice were obtained from the Centro de Bioterismo, FMUSP. Nod-scid mice (NOD/LtSz-Prkdc^{scid}, 8 week old, female) were obtained from UNIFESP. All animals were maintained in SPF conditions, with food and water *ad libidum*.

All procedures and conditions were approved in accordance to the guidelines of animal care and use by the Scientific and Ethics Committee of the Instituto do Coração, FMUSP.

Cell transduction and vaccine protocol

The vaccine protocol was divided in three steps: (i) *ex vivo* transduction, (ii) vaccination and (iii) challenge. For the first, B16 cells ($1.2x10^{6}$) were transduced with the vectors AdPGLUC (MOI 1800), AdPGp19 (MOI 900), AdPGIFNβ (MOI 900), or with the combination of AdPGp19 and AdPGIFNβ (MOI 900 for each one) in 10 cm dishes with 2 mL medium for 4 hours before the addition of 8 mL of fresh medium. Cells were then incubated for 48 hours, trypsinized, washed once with PBS and counted (viable and dying together). In the second step, these cells were inoculated (s.c) in the left flank (denominated hereafter as vaccine site) of naïve immunocompetent C57Bl/6 mice and 7 days after the last vaccine, in the last step, these animals were challenged with fresh B16 cells ($1x10^{5}$) inoculated (s.c) in the right flank (denominated challenge site). Additionally, mice were also vaccinated with cells that were previously transduced *ex vivo* with the AdPGLUC vector and killed with three cycles of freeze-thaw

(B16+LUC group). Tumor progression was accompanied and tumor volume calculated as described previously (9). As specified in the results section, different cell quantities and vaccination regimens were also used. For the therapeutic vaccine model, naïve C57Bl/6 mice were inoculated (s.c) with fresh TM1 cells ($8x10^4$) in the right flank and 7 days later vaccinated in the left flank (s.c) with TM1 cells ($4x10^5$) that were transduced *ex vivo* with the vectors AdRGDPGIFNβ (MOI 500), or with the combination of AdRGDPGp19 and AdRGDPGIFNβ (MOI 500 for each one). In this case, the vectors contain an RGD tripeptide modification that enhances the efficiency of delivery and will be described elsewhere (manuscript in elaboration).

CD45 immunochemistry

Challenge tumors were collected on day 16 after challenge and embedded in tissue-freezing medium (Tissue-Tek/OCT™), cut in sections and mounted on poly(L-lysine) coated slides (Sigma). For the immunochemistry reaction slides were blocked with 1% bovine serum albumin (30 min), incubated (1 hour, RT) with a purified rat anti-mouse CD45 antibody (1/100, BD Pharmigen) followed with Biotin Mouse Anti-Rat IgG2b (1/200, BD Pharmigen) and using the DAB substrate kit (BD Pharmigen). Slides were visualized by light microscopy, and 5 fields for each slide were randomly photographed and positive cells counted with the assistance of ImageJ software (NIH).

Cell cycle analysis

Cells were transduced as described above and cell cycle analysis done as per Merkel et al (1). Briefly, after treatment cells were incubated in PBS containing propidium iodide and RNAse. After washing, cells were submitted to fluorescence measurement by flow cytometry (FACScan, Becton-Dickenson) and the cell cycle profile analyzed by CellQuest software (BD, USA).

Clonogenic assay

After transduction as described above, 1000 cells were plated in 10 cm dishes, maintained in culture for 12 days and then fixed with acetic acid/methanol 1:7 (v/v), washed once with PBS (10 mL) and then incubated with a 0.5% crystal violet solution for 2 hours.

RT-qPCR

B16 cells were transduced as described above and after 48 hours, mRNA was collected to perform qPCR analysis as described in detail in (1). All samples were tested in triplicate and analyzed by the 7500 Fast Software, version 2.05 (Applied Biosytems). The $2^{-\Delta\Delta Ct}$ method was used for gene expression quantification and data is presented as

fold change in expression (log2) as compared to the non-transduced B16 condition. Primers are described in **Supplemental table 1**.

Priming, cytotoxic assay and bead array

B16 cells ($3x10^5$) were transduced as described above and, after 48 hours, injected into the footpad of C57Bl/6 mice. Ten days later, cells were collected from popliteal lymph nodes and co-cultured with fresh B16-LUC cells (stably modified to express luciferase) at different ratios (1:1, 1:10 and 1:20, B16: popliteal lymph node cells) for two days in a round bottom 96 well plate. In order to evaluate cytokine production, the supernatant from the 1:20 condition was collected and subjected to cytometric bead array (CBA; BD Bio-sciences) on a FACSCaliber cytometer equipped with CBA software (BD Bio-sciences) according to manufacturer's instructions. The luciferase activity of the adhered cells was measured with Dual-Glo Luciferase Assay System (Promega) following the manufacturer's instructions and using a luminometer (Victor, Perkin-Elmer, USA).

Immune suppression

Immunocompetent C57Bl/6 mice were immunosuppressed with dexamethasone as described in (11). Briefly, the mice were injected in the right flank (s.c) with 3 or 10 mg/kg/day of dexamethasone (Roche) or PBS (Control group) for 19 consecutive days. On the eighth day of treatment, these mice were inoculated (s.c) in the left flank with B16 cells ($1x10^5$) transduced as described above and on the twelfth day peripheral blood from the LUC, LUC+3DEX and LUC+10DEX groups was collected by retro-orbital puncture to count white blood cells at the FMUSP.

$CD4^+$ and $CD8^+$ T lymphocyte depletion

In vivo depletion of $CD4^+$ or $CD8^+$ T cells was performed by treating (i.p) vaccinated mice with ascites containing GK1.5 or 53.6.7 rat IgG antibodies, respectively, kindly provided by Dr. Mauricio Martins Rodrigues (UNIFESP). The control group was treated with ascites containing the UF5H2 anti-CEA, IgG1 antibody (a human melanoma antigen) kindly provided by Dr. Roger Chammas (FMUSP). To deplete $CD4^+$ lymphocytes, mice were injected after the first vaccine on day 9, 11, 13 and 21 after first vaccination. To deplete $CD8^+$ lymphocytes, depletion started two days before vaccination and continued on days 4 and 6. Additionally, on day 16, one more injection was made to maintain the depletion. The efficacy of these protocols was superior to 96% and confirmed in spleen cells and inguinal lymph nodes by FACS analysis using the following antibodies AcαCD3 (PE, 17A2, BD Bioscence), AcαCD4 (PECy7, RN4.5, Invitroven) and AcαCD8 (APC, 5H10, Invitrogen).

Analysis of tumor infiltrating CD4+ and CD8+ T Lymphocytes

C57Bl/6 mice were vaccinated ($3x10^5$ B16 cells, 1X) and challenged as explained above and 18 days after challenge tumors were collected, dissociated in Liberase (35μg/ml, Roche) and cells were analyzed by flow cytometry (FACScan, Becton-Dickenson) for the expression of CD3, CD4 and CD8 (using the antibodies described above).

Statistical Analysis

Data is presented as mean ± SEM. Statistical differences between groups was indicated with p-values, where *p<0,05, **p<0,01 and ***p<0,001. Comparisons between two groups an unpaired t-test was used. If more than two groups were compared, results were analyzed by 1-way ANOVA followed by Tukey post-hoc test. Statistical analysis for tumor progression curves was performed by Two-way ANOVA and Bonferroni post-test. For survival or tumor-free mice, Log Rank Mantel-cox test was performed, followed by Wilcoxon test. All analyses were made using the GraphPad Prism 5 software.

Results

Vaccination with cells dying due to treatment with the $p19^{Arf}$ and IFNβ combination induces a protective anti-tumor immune response

In order to investigate if the combined gene transfer of p19Arf and IFNβ could induce an anti-tumor immune response, we developed a prophylactic vaccine tumor protocol **(Fig 1a)**. In this model, the B16 cell line is transduced *ex vivo* and while dying (when cell death is apparent upon cell cycle analysis, but has not yet reached its maximum, **Fig 1b**) is used as the immunizing agent against a subsequent tumor challenge. In controls groups, mice were vaccinated with PBS, live B16 cells (to investigate the influence of a tumor at the vaccine site on the progression at the challenge site) or dead B16 cells that were transduced *ex vivo* with AdPGLUC vector (to control for the presence of tumor and viral antigens). As hoped, at the challenge site, only the group that received the $p19^{Arf}$ and IFNβ vaccination showed a significant reduction in tumor volume (66.5 mm^3) when compared to control groups, such as Dead B16+LUC (394 mm^3) **(Fig 1c)**. Challenge tumors were collected and analyzed for the presence of the common leucocyte antigen (CD45) as a preliminary examination of the involvement of the immune system. Indeed, an increase of $CD45^+$ cells was observed only in the p19+IFNβ challenge tumors **(Fig 1d)**. Despite the induction of cell death by the $p19^{Arf}$ and IFNβ combination, B16 cells still formed tumors at the vaccine site. Interestingly, tumor formation was significantly delayed **(Fig 1e)** and progression

significantly reduced **(Supplemental figure 1)** in the p19+IFNβ group as compared to the Live B16 group.

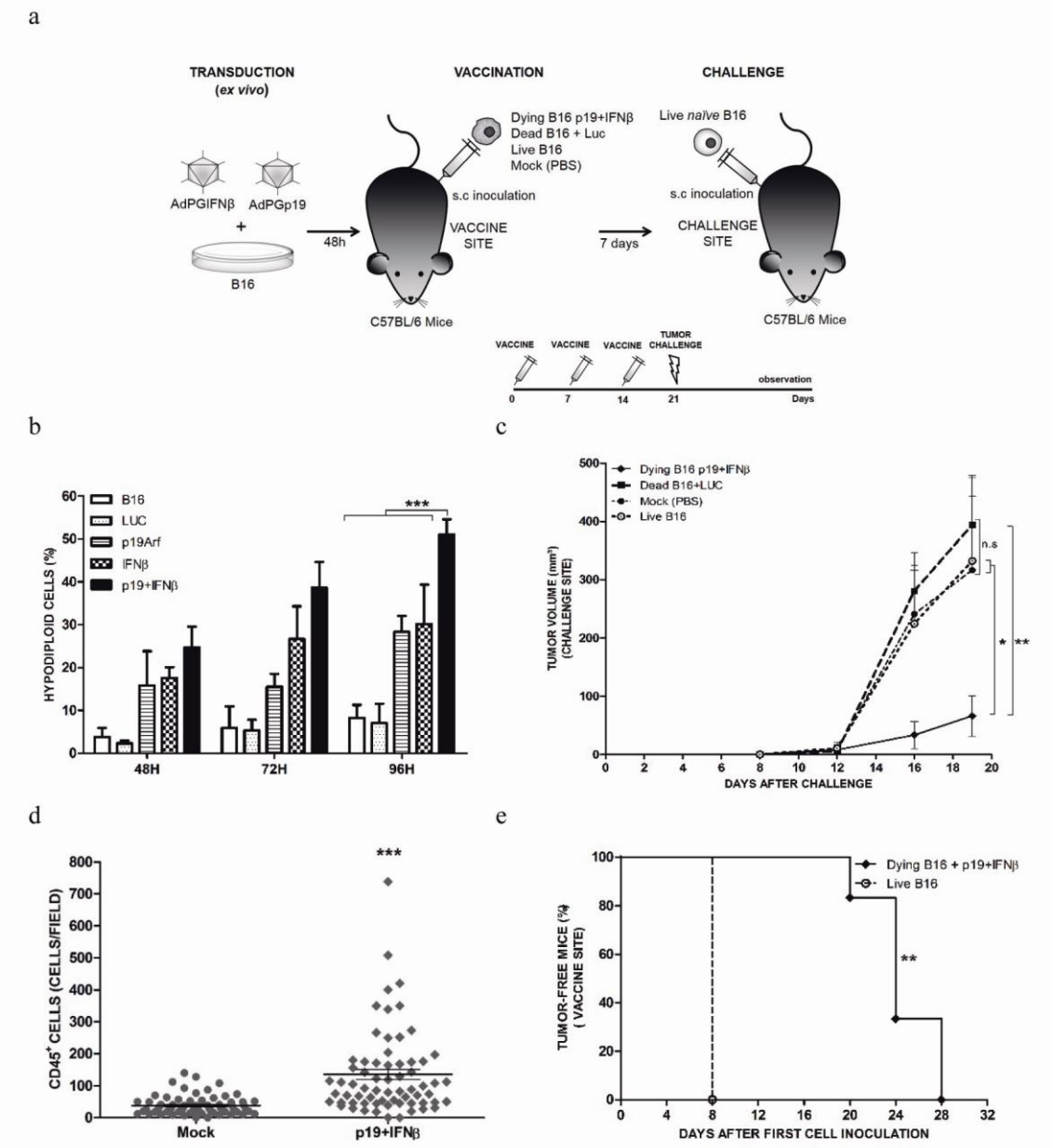

Figure 1. Vaccination with cells dying due to treatment with the p19Arf and IFNβ combination induces a protective anti-tumor immune response. (a) Schematic representation of the vaccination protocol. C57Bl/6 mice were inoculated (3x, once per week) in the vaccine site with PBS, Dead Cells transduced with the vector AdPGLUC, Live cells (only 1 application with 5x105 cells at day 14) or B16 cells co-transduced with the vectors AdPGp19 and AdPGIFNβ (3x105 cells). On day 21, the animals were challenged. **(b)** Cell cycle analysis of transduced B16 cells reveals kinetics of hypodiploid population *in vitro*. **(c)** Tumor progression at the challenge site. **(d)** Immunohistochemical analysis of CD45+ infiltrating cells in the challenge tumors. **(e)** Tumor onset at the vaccine site. Dying B16 p19+IFNβ (n = 5); Dead B16+ LUC (n = 5); Mock (n = 5); Live B16 (n = 3).

Conditions for tumor development at the vaccine site

To investigate the possible conditions that give rise to the tumors at the vaccine site, we first performed a clonogenic assay to reveal the resistance of B16 cells to treatment. Treatment with $p19^{Arf}$ or its combination with IFNβ drastically decreased the number of colonies formed, yet resistant clones were observed **(Fig 2a).** Next, we

assessed the influence of the amount of cells and the number of applications used during the vaccination with the expectation of finding limits for tumor formation at the vaccine site. For this, treated B16 cells were inoculated (s.c) with different quantities (10^5 or $3x10^5$) in a single injection or divided in three weekly injections **(Fig 2b)**. Cells treated by the p19+IFNβ combination tend not to develop tumors when inoculated in a single injection, regardless of the amount of cells used. Yet, when the same quantity of cells was divided in three injections, tumors were formed, as observed in the $3x10^5$ (1X) and 10^5 (3X) groups. Additionally, tumors appeared faster when larger quantities of cells were applied, compare $3x10^5$ (3X) and 10^5 (3X) **(Fig 2c)**. Thus, by altering the inoculation regimen, long lasting tumor-free survival was achieved.

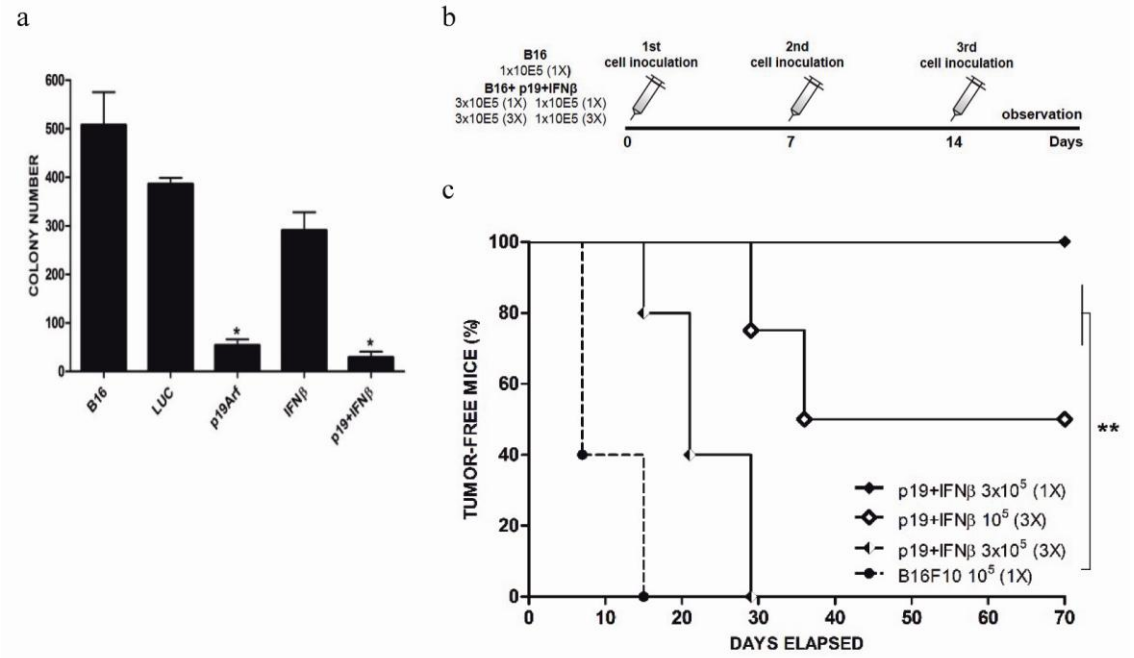

Figure 2. Conditions for tumor development at the vaccine site. (a) Clonogenic assay of treated B16 cells. Data from 3 independent experiments. **(b)** Schematic representation of the cell inoculation protocol. B16 cells were co-transduced with the vectors AdPGp19 and AdPGIFNβ and later, animals were inoculated with 10^5 or $3x10^5$ cells in a single injection or divided in three injections, one each 7 days. **(c)** Tumor onset in the inoculated mice. n=5 for all groups.

Treatment with $p19^{Arf}$ and IFNβ abrogates tumor formation in hosts with normal NK activity and up-regulates ULBP1 NK ligand, IL-15 and death receptors

We speculated that the rejection of the $p19^{Arf}$ and IFNβ treated cells at the vaccine site would involve not only the induction of cell death, but also the immune system. To address this question, we inoculated (s.c) IFNβ or $p19^{Arf}$ and IFNβ treated cells in the hosts C57Bl/6 (immune competent), Nude (adaptive immune deficient) and Nod-scid (innate and adaptive immune deficient) **(Fig 3a)**. Strikingly, p19+IFNβ tumors

did not grow in either the C57Bl/6 or Nude mice, which retain Natural Killer (NK) cell activity (12), but did grow in the Nod-scid strain, known for having low NK activity (13). The treatment of cells with just IFNβ was not able to abrogate tumor formation in any host, growing in 40 to 60% of the animals of each group **(Fig 3b)**. In order to corroborate this observation, we immune suppressed C57Bl/6 mice with 3 or 10 mg/kg of dexamethasone, as per Keil et al (11) who showed that mice treated with dexamethasone were less resistant to B16 tumor formation due to the reduction in NK cell activity. Indeed, in the p19+IFNβ combination group, immunosuppression with 10 mg/kg of dexamethasone was associated with tumor formation (60% as compared to the immune competent condition). The dose of 3mg/kg did not alter tumor formation in the combination group. In striking contrast, tumor formation in the IFNβ group was accelerated in all conditions tested **(Fig 3c)** while immunosuppression did not have an impact on the already fast growing AdPGLUC control **(Supplemental Figure 2a).** To confirm immunosuppression, white blood cells were counted, revealing a decrease in the percentage of lymphocytes and eosinophils below the reference values. Yet, the neutrophil population grew, elevating the total number of leucocytes **(Supplemental Figure 2b)**.

Moreover, genes involved in NK cell migration (CXCL2, CCL2, CCL3, CCL4, CXCL1), activation (RAET-1E, RAET-D, ULBP-1, H60, IL-15) and cell death (KILLER/DR5, FAS/APO1) were analyzed in B16 cells treated with just p19Arf, IFNβ or the combination. A significant up regulation of the IL-15 cytokine, ULBP1 NK ligand and both FAS/APO1 and KILLER/DR5 death receptors was observed only in the combination treatment, suggesting a mechanism for NK cell activation **(Fig 3d)**. The other genes investigated, including all of the chemokines, were not up regulated after treatment **(Supplemental Figure 2c)**. These assays indicates that cells treated by the p19+IFNβ combination are rejected trough a NK-mediated immune response and suggests that, though rare, B16 cells escaping both the treatment and the NK cell activation can form tumors at the vaccine site.

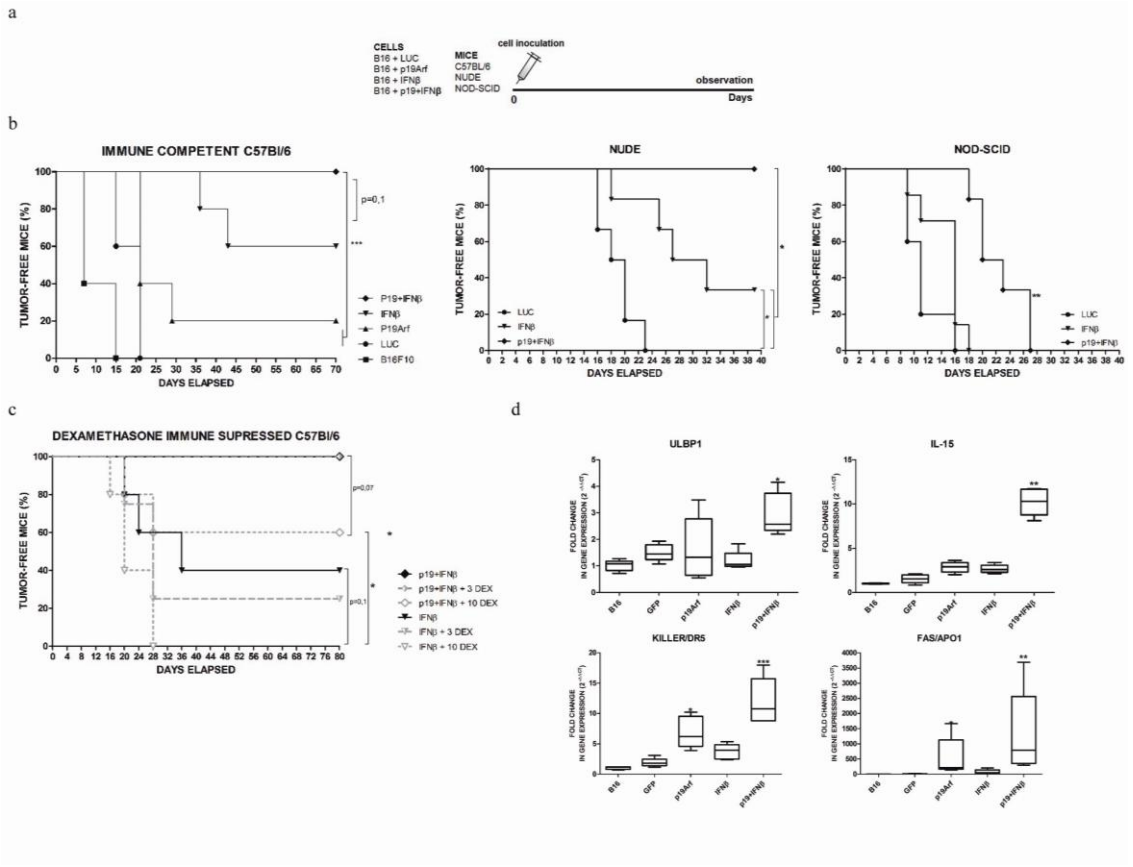

Figure 3. Treatment with p19^{Arf} and IFNβ abrogates tumor formation in hosts with normal NK activity and up-regulates ULBP1 NK ligand, IL-15 and death receptors. (a) Schematic representation of cell inoculation protocol. B16 cells transduced *ex vivo* with the adenoviral vectors AdPGLUC, AdPGp19, AdPGIFNβ or the AdPGp19/AdPGIFNβ combination and later inoculated (s.c) in a single application with 1x10^5 cells in C57Bl/6, Nude or Nod-scid mice. **(b).** Tumor onset in the inoculated mice. n=6 for all groups. **(c)** Tumor onset in the dexamethasone immunosuppressed C57Bl/6 mice. Immune competent mice were injected (s.c) daily for 16 consecutive days with 3 or 10mg/kg dexamethasone and on the eighth day implanted with the treated B16 cells. n=5 for all groups. **(d)** RT-qPCR analysis of gene expression in B16 cells that had been transduced *ex vivo*. Cells were transduced and incubated for 48 hours before collected for RT-qPCR. β-actin was used as the reference gene. Data was calculated using $2^{-\Delta\Delta Ct}$ method and presented as fold change (log2) as compared to the non-transduced B16 condition. Data from derived from 5 independent experiments.

IFNβ alone or in combination with p19^{Arf} induces a TH1 immune response

We next employed the prophylactic vaccine model in order to investigate the ability of single or combined treatments to unleash a protective immune response against a tumor challenge **(Fig 4a)**. At the challenge site, tumor progression was markedly reduced to a similar extent with either IFNβ (104 mm^3) or its combination with p19^{Arf} (112 mm^3). *Ex vivo* treatment with just p19^{Arf} (256 mm^3) conferred no significant protection as compared to the control group (586 mm^3) **(Fig 4b)**. Interestingly, the p19+IFNβ treatment was more frequently associated with tumor-free progression at the vaccine site as compared to all other conditions **(Supplemental Figure 3a)**, thus conferring a survival benefit exclusively for this group **(Fig 4c)**. Alternatively, transduced B16 cells were implanted in the foot pad of C57Bl/6 mice,

popliteal lymphocytes isolated and co-cultured with B16-LUC (stably modified to express luciferase) to evaluate their cytotoxic activity and cytokine expression. Bead Array analysis revealed in both the IFNβ and the combination groups an increase of cytokines associated with a TH1 immune response (IL-12, IL-6, TNF-α, IFN-γ, MCP1/CCL2) **(Fig 4d)** and, with the exception of IL-10, no alteration was seen in the TH2 and TH17 profile **(Supplemental Figure 3b)**. In corroboration, a significant difference in luciferase activity, used to indicate viable tumor cells, was also only observed in those groups where IFNβ was included **(Fig 4e)**, indicating that immune protection is dependent on IFNβ.

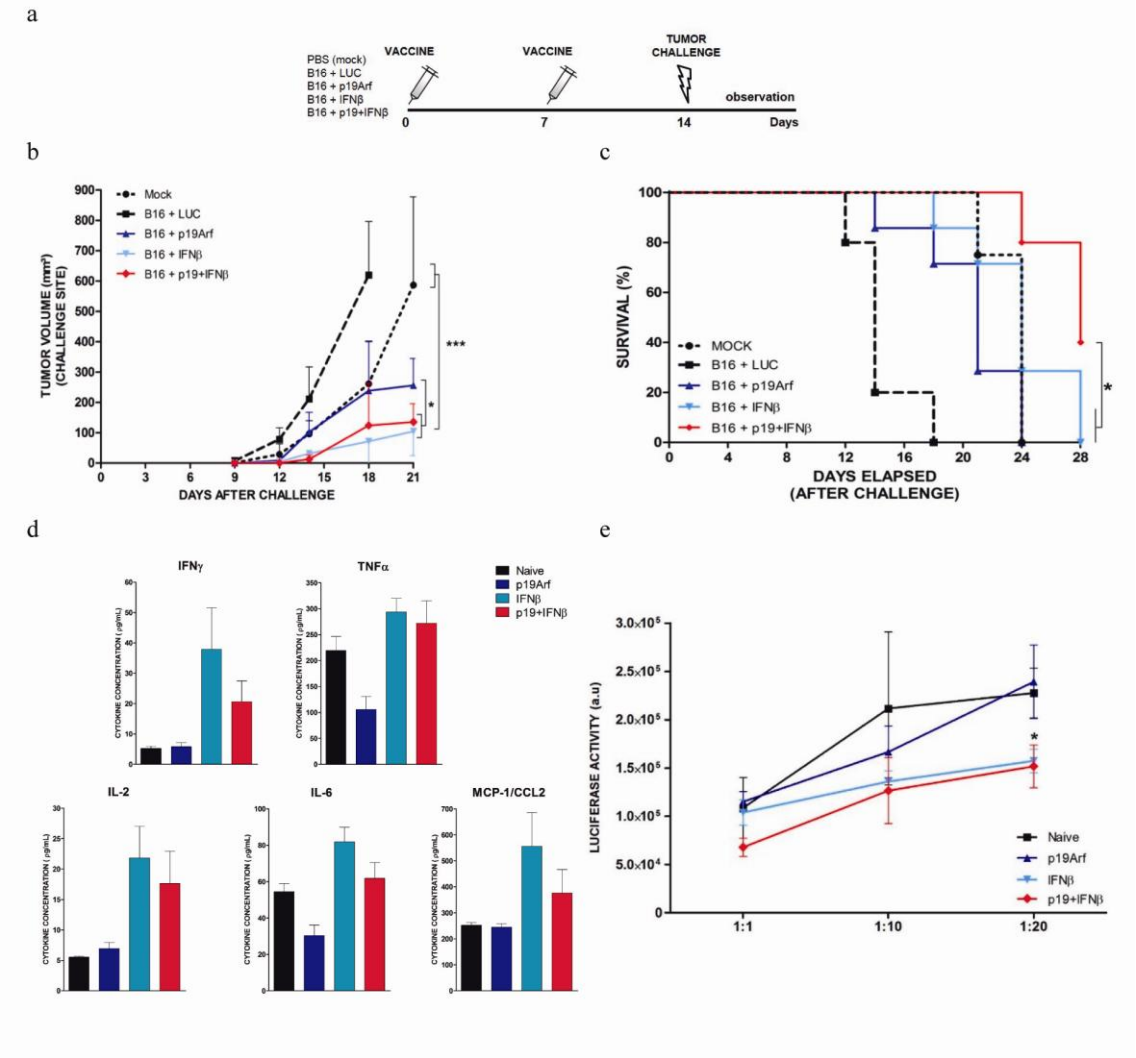

Figure 4. IFNβ alone or in combination with p19^{Arf} induces a TH1 immune response. (a) Schematic representation of the vaccination protocol. C57Bl/6 mice were vaccinated (2x, once a week) with B16 cells transduced *ex vivo* with AdPGLUC, AdPGp19, AdPGIFNβ or the AdPGp19/AdPGIFNβ and on the 14 day mice were challenged. **(b)** Tumor progression at the challenge site. **(c)** Survival curve of the vaccinated mice. n=4 for the Mock and B16+LUC groups. n=6 for the B16+p19+IFNβ. n=7 for the IFNβ group. **(d)** Cytokine expression analysis. **(e)** Luciferase activity of adherent B16 cells. n=4 for the Mock group, n=5 for the p19^{Arf} and p19+IFNβ groups and n=6 for the IFNβ.

Both $CD4^+$ and $CD8^+$ T lymphocytes are necessary for immune protection

To uncover the role of Helper ($CD3^+CD4^+$) and Cytotoxic ($CD3^+CD8^+$) T lymphocytes, first we analyzed the infiltration of CD4+ and CD8+ T Lymphocytes in the challenge tumor 18 days after vaccination, revealing a significant increase in the percentage of both of these lymphocytes in comparison with mice in the mock treatment group **(Fig 5a and 5b).** Moreover, depletion of $CD4^+$ or $CD8^+$ T cells was performed in the prophylactic vaccination model by injection (i.p) of monoclonal antibodies and growth of the challenge tumor was monitored. Depletion of $CD4^+$ cells after immunization revealed these cells to be critical for the protective effect, since tumors of this groups were equally as large as those in the PBS group and significantly bigger than the $p19^{Arf}$ and IFNβ groups **(Fig 5c)**. In sharp contrast, depletion of $CD8^+$ cells did not affect tumor growth in this approach (data not shown). However, if the depletion protocol was performed during the immunization step (not after), the loss of $CD8^+$ population was shown to be critical since the vaccine effect was completely abolished **(Fig 5d)**. No difference in progression at the vaccine site was noticed in either protocol (data not shown). Taken together, these results indicate that the participation of $CD4^+$ and $CD8^+$ T lymphocytes is fundamental for combating the tumor at the challenge site.

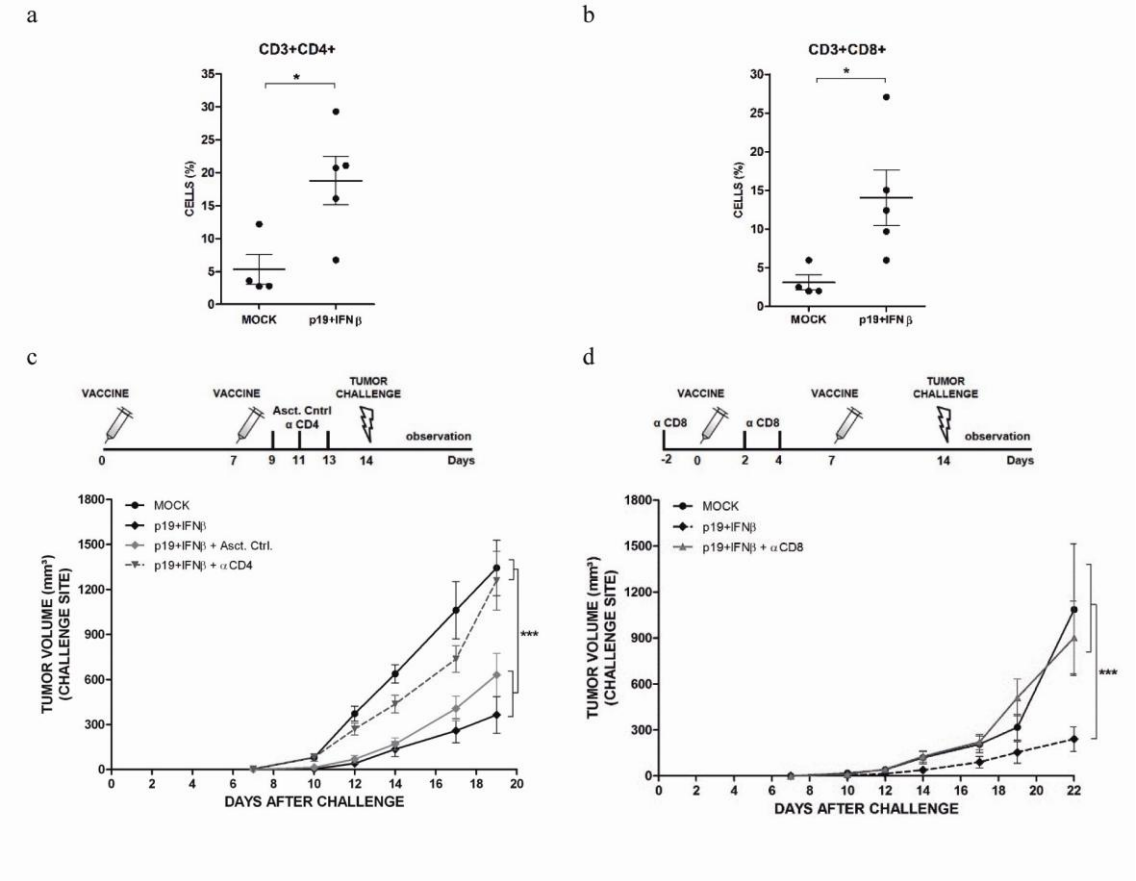

Figure 5. Both CD4$^+$ and CD8$^+$ T lymphocytes are necessary for immune protection. Analysis of tumor infiltrating CD3+CD4+ **(a)** and CD3+CD8+ **(b)** lymphocytes in challenge tumors. Progression of challenge tumors upon depletion of CD4$^+$ T cells **(c).** Progression of challenge tumors upon depletion of CD8$^+$ T cells **(d)**. Vaccination was made as described above. n=6 for all groups.

The p19^{Arf} and IFNβ combination as a cancer immunotherapy strategy

The putative vaccine involving the use of cells dying due to the treatment with the combination of p19^{Arf} and IFNβ was next tested in experimental models of immunotherapy. First, we addressed the duration of protection in the prophylactic model, but with just one application of the vaccine and with the tumor challenge performed 73 days later **(Fig 6a)**. Challenge tumor progression was still reduced even 73 days after vaccination with 10^5 (128 mm^3) or $3x10^5$ cells (72 mm^3), but not with $5x10^4$ cells (1,411 mm^3), suggesting that an immunological memory was created and that a minimum number of cells was needed to induce protection in this setting **(Fig 6b)**. Next, using a different mouse melanoma cell line, called TM1, our vaccine was evaluated in a therapeutic application. To this end, mice were first inoculated with naïve TM1 tumor cells and, 7 days later, vaccinated with a single inoculation of cells treated with just IFNβ or its combination with p19^{Arf} **(Fig 6c)**. Neither of the groups developed tumors at the vaccine site, but only the animals vaccinated with cells treated by the combination had reduced tumor progression at the challenge site (160 mm^3), even when compared to the IFNβ group (316 mm^3). Thus, the TM1 therapeutic vaccine model not only revealed effectiveness of our approach in a second cell line, but also indicated that the combined treatment was superior in providing immune protection in a more advanced stage of tumor progression **(Fig 6d)**.

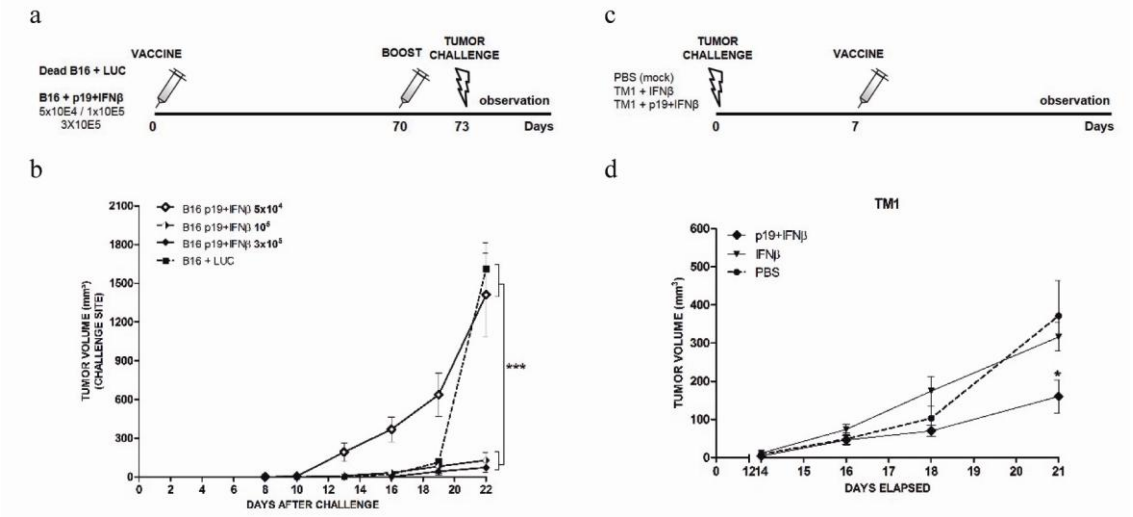

Figure 6. The p19^{Arf} and IFNβ combination as a cancer immunotherapy strategy. (a) Schematic representation of the late challenge. C57Bl/6 mice were inoculated (single application) in the vaccine site with PBS, or B16 cells co-transduced with the vectors AdPGp19 and AdPGIFNβ ($5x10^4$, $1x10^5$ or $5x10^5$ cells). Seventy days later, a boost vaccination was made with newly treated cells ($5x10^4$ cells) and three days later (73 days since the start of the protocol) challenge with naïve B16 cells. **(b)** Tumor progression

curve in the challenge site. n=4 for the Dead B16+LUC. n=5 for all the others groups. **(c)** Therapeutic vaccine schematic representation. On day 0, C57Bl/6 mice were inoculated with naïve TM1 tumor cells and 7 days later, vaccinated with a single inoculation of TM1 cells treated with IFNβ or its combination with $p19^{Arf}$. **(d)** Tumor progression in the tumor inoculation site. n=6 for all groups.

Proposed model

Based upon the results presented here and in previous studies, we propose the following mechanism for this vaccine approach **(Supplemental Figure 4)**: First, *ex vivo* co-transduction of B16 cells with both AdPGp19 and AdPGIFNβ adenoviral vectors reestablishes the p53 antitumor pathway and triggers apoptosis (confirmed by cleavage of caspase 3 and exposure of annexinV) (9). Next, in the vaccination step, these dying cells also present up-regulation of the NKG2D NK ligand, IL-15 as well as cell death receptors Killer/DR5 and Fas/APO1, contributing to the activation of NK cells and rejection of the tumor at the vaccine site. Along with the combat of vaccine cells, we speculate that release the of cell death associated molecules (CDAM), secretion of IFNβ and exposure of tumor antigens are perceived by antigen presenting cells of the adaptive immune system, unleashing a TH1 cytotoxic immune response. Finally, naïve tumor cells at the challenge site are attacked by $CD8^+$ and $CD4^+$ T lymphocytes that reduce tumor progression, thus prolonging survival of the mouse.

Discussion

We have shown that the vaccination using cells dying due to the treatment with $p19^{Arf}$ and IFNβ controls outgrowth at the vaccine site and also an immune response that reduces tumor progression in both prophylactic and therapeutic models. We propose that, by occurring *in vivo*, the process of cell death may also contribute to the immunogenity of our vaccine through the release of CDAM , as demonstrated that tumor cells can die in an immunogenic cell death (ICD) context (14, 15). To be classified as an inducer of ICD, the operational definition is: *ex vivo* treatment must induce prophylactic immune protection in a vaccine model and, therapeutic effects (i.e reduction of tumor growth) must depend at least in part on the immune system (14). We believe that our results satisfy this definition since immune protection was induced by the $p19^{Arf}$ and IFNβ vaccine, reducing tumor progression at the challenge site due to the involvement of $CD4^+$ and $CD8^+$ T lymphocytes.

Moreover, our results suggest that rejection of $p19^{Arf}$ and IFNβ treated cells at the vaccine site involves NK cell activity. Additional evidence came from the dexamethasone immune suppressed mice that developed more tumors than their immune competent counterpart. In the work of Keil and colleagues (11), dexamethasone

treated mice lost their resistance to B16 tumors due to a reduction in the lytic activity of NK cells . As in theirs, our work has also revealed a reduction in the number of lymphocytes and an increase in neutrophils, thus supporting that our suppression regime was reliable. In addition, a possible advantage of inducing NK cells at the vaccine site would be their ability to collaborate with DC to promote a TH1 immune response, thus assuming a helper phenotype (16-18).

Upon further investigation, we found that only combined treatment $p19^{Arf}$ and IFNβ up regulated the expression of the ULBP-1 NK ligand, the IL-15 cytokine and death receptors, factors known to influence NK response. Other studies have demonstrated a role for p53 pathway in NK mediated immunity by up regulating ULBP1 and ULBP2 NKG2D ligands (19, 20). However, to the best of our knowledge, no other work has related the up regulation of IL-15 by p53, yet type I IFN is known to induce expression of this cytokine (21). Interestingly, IL-15's strong immune-enhancing activity is being increasingly recognized in NK cell immunotherapy, as it stimulates survival, maturation and effector functions (22-24).

The prophylactic protection at the challenge site was more dependent on the immune modulatory effects of IFNβ than treatment with $p19^{Arf}$. We suspect that difference on the dynamics of IFNβ expression may impact the vaccine protection. The expression of IFNβ is lost when cells treated by the combination die, yet cells treated only with IFNβ survive much longer, creating an opportunity for prolonged expression and interaction with the immune system *in vivo*. Indeed, it has already been reported that timing and magnitude of type I interferon responses impact CD8+ T cell response (25). Nevertheless, in the therapeutic vaccine model, only the combination brought a reduction in tumor progression, indicating that it is indeed superior to the IFNβ mono-treatment. However, it is not yet clear if the $p19^{Arf}$ and IFNβ combination can stimulate a superior T lymphocyte response than IFNβ alone.

One possible translational scenario would be an autologous vaccine in which melanoma cells obtained from surgery could be transduced *ex vivo* and returned to the patient in order to activate the immune system, combatting tumor regrowth and metastasis. Though not studied here, further development, such as irradiation of the cellular vaccine, may be required to ensure the safety of this approach. The use of a vaccine, instead of in situ gene therapy, may have the added advantage of restoring tumor cell immunogenicity in a non-immunosuppressive microenvironment, thus facilitating immune recognition of treated cells, antigens and the generation of an antitumor immune response. Additional benefit may come from the association of our

vaccine approach with other treatments, especially those that enhance the immune response, thus increasing efficacy. Though much work remains to be done, we believe that development of this novel immunotherapy strategy is warranted given the encouraging data that has been presented here.

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AUTHOR CONTRIBUTIONS

RFVM and BES conceived and designed the experiments, as wells as wrote the manuscript. RFVM also analyzed the data and performed the experiments in Figures 1, 2, 3, 4, 5, 6. JPPC discussed the data, performed experiment in Figure 1 d and assisted in Figures 4 d and e. AH provided RNA and performed together with RFVM qPCRs in figure 3 d. STL assisted on figure 4 d. CAM provided aliquots for virus production. ECS provided material, infrastructure and housed Nude mice for depleting antibody production. BES is the laboratory leader and supervised the study.

5

Intratumoral immunization by p19Arf and interferon-beta gene transfer in a heterotopic mouse model of lung carcinoma

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Intratumoral Immunization by p19Arf and Interferon-β Gene Transfer in a Heterotopic Mouse Model of Lung Carcinoma[1,2]

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Abstract

Therapeutic strategies that act by eliciting and enhancing antitumor immunity have been clinically validated as an effective treatment modality but may benefit from the induction of both cell death and immune activation as primary stimuli. Using our AdRGD-PG adenovector platform, we show here for the first time that *in situ* gene transfer of p19Arf and interferon-β (IFNβ) in the LLC1 mouse model of lung carcinoma acts as an immunotherapy. Although p19Arf is sufficient to induce cell death, only its pairing with IFNβ significantly induced markers of immunogenic cell death. *In situ* gene therapy with IFNβ, either alone or in combination with p19Arf, could retard tumor progression, but only the combined treatment was associated with a protective immune response. Specifically in the case of combined intratumoral gene transfer, we identified 167 differentially expressed genes when using microarray to evaluate tumors that were treated *in vivo* and confirmed the activation of CCL3, CXCL3, IL1α, IL1β, CD274, and OSM, involved in immune response and chemotaxis. Histologic evaluation revealed significant tumor infiltration by neutrophils, whereas functional depletion of granulocytes ablated the antitumor effect of our approach. The association of *in situ* gene therapy with cisplatin resulted in synergistic elimination of tumor progression. In all, *in situ* gene transfer with p19Arf

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[2] Author contributions: J. P. P. C., R. V. F. M., and B. E. S. conceived and designed the experiments; J. P. P. C., R. V. F. M., A. H., and S. A. performed the experiments; J. P. P. C., R. V. F. M., P. D. V., and B. E. S. analyzed the data; S. A., G. K., D. B. Z., and E. C. S. contributed reagents, materials, and analysis tools; J. P. P. C. and B. E. S. wrote the manuscript.
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and IFNβ acts as an immunotherapy involving recruitment of neutrophils, a desirable but previously untested outcome, and this approach may be allied with chemotherapy, thus providing significant antitumor activity and warranting further development for the treatment of lung carcinoma.

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Introduction

Despite extraordinary advances in controlling cancer progression, long-term benefit remains disappointing [1–4]. Even so, current treatments do extend survival and create new therapeutic opportunities, especially for strategies that induce a long-lasting response, such as the modulation of antitumor immunity [5]. Several findings support the hypothesis that local immunomodulation is able to control and even eradicate primary and distant tumors [6–9]. In addition, studies have shown that effective T-cell activation can be achieved intratumorally, dispensing the participation of draining lymphatic organs [10,11]. In this regard, we investigated whether intratumoral gene transfer of p19Arf and interferon-β (IFNβ) would generate immunogenic cell death (ICD) in a strong immunostimulatory context, allowing the activation of a cellular antitumor response.

The p19Arf (alternative reading frame) tumor suppressor protein is well known as an inhibitor of the Mdm2-mediated ubiquitination of p53, thus contributing to the activation of p53 in response to cellular stress [12,13]. Considering the pivotal role of p53 signaling in cancer prevention, mutations in this pathway are a very common event in cellular transformation. Deregulation of the p53 pathway is also associated with increased resistance to chemo- and radiotherapy [14,15]. In this context, restoration of the p53 pathway has been extensively explored in preclinical and clinical settings [16–18]. P19Arf gene transfer has been shown to inhibit cell growth and induce apoptosis in several models [19–23]. Furthermore, p19Arf has been implicated in antitumor functions independently of p53 activation [17].

The multifunctional cytokine IFNβ has been implicated in the stimulation of a plethora of genes which impact virtually the entire cellular organization [24]. In cancer research and therapy, type I IFNs, specifically α and β, are extensively used due to their tumor suppressor capabilities by acting directly on tumor cells and through immunomodulatory properties. Type I IFNs induce apoptosis and cell cycle arrest in several models [25–27]. Interestingly, IFNβ can promote p53 transcription, enhancing cell death in response to DNA-damaging agents [28]. In addition to the direct effects of type I IFNs on cancer cell viability, an impressive number of studies have shown the ability of type I IFN to modulate the antitumor immune response. Type I IFNs are implicated in recruitment, proliferation, differentiation, and activation of immune cells [29–32]. Curiously, recent evidence from mouse models has shown that IFNβ signaling plays a pivotal role in the antitumor response induced by radiotherapy [33,34] and by chemotherapy with anthracyclines [35]. IFNβ gene transfer has been exploited in a considerable number of studies, indicating that IFNβ is sufficient to modulate the tumor microenvironment, inducing or improving immunological response [36–40].

We have previously shown that intratumoral gene transfer of p19Arf and IFNβ or IFNβ alone is able to reduce tumor growth in a murine model of melanoma. Although IFNβ gene transfer alone could produce this effect, only its association with p19Arf induced increased cell death *in vivo* [23]. In vaccination strategies, we also showed that B16 transduced with the combination of p19Arf and IFNβ prolonged survival in mice [41]. Until now, we had no evidence that our gene transfer approach, when applied directly to the tumor mass, would induce a protective immune response. Here we demonstrate for the first time that combined *in situ* gene therapy is able to circumvent tumor suppressive microenvironment and provide a significant advantage for immunostimulation. This advantage is characterized by differential expression of genes that affect the immune response and chemotaxis, validated through the observation of recruitment of neutrophils and antitumor activity of granulocytes. The combined gene transfer approach also yielded a synergistic impact on tumor growth when associated with cisplatin.

Materials and Methods

Mice and Cell Line

Wild-type, female C57BL/6 mice were purchased from Centro de Bioterismo, Faculdade de Medicina, Universidade de São Paulo (FM-USP). Animals were 6 to 9 weeks of age at the time of experimental procedures. All experiments were approved by the Ethics Committee of FM-USP (research protocol 116/10).

Lewis lung carcinoma cell line (LLC1) was kindly provided by Dr. Karim Benihoud and cultured in Dulbecco's modified Eagle medium (Gibco cat. no. 12,100–046) supplemented with 10% of fetal bovine serum and Gibco Antibiotic-Antimycotic solution.

Adenovector Production

The serotype 5 adenovectors used in this work contain the RGD modification in the fiber protein [42]. The backbone provided by Dr. Hiroyuki Mizuguchi (Osaka University, Japan) was modified to allow *in vitro* recombination using clonase (Invitrogen, 12538120) (A.H., manuscript submitted). Adenovectors express eGFP, IFNβ, and p19Arf under the control of a p53 responsive promoter [43] or LacZ the under control of the CMV promoter.

Adenovector production was performed by transfection of linearized plasmids into HEK293A cells followed by amplification cycles and purification using iodixanol gradient [44]. Purified adenovectors were stored in PBS 7% glycerol at −80°C. Titration was done using Adeno-X Rapid Titer Kit (Clontech, 632250).

In Vitro Experiments

LLC1 cells were transduced with adenovector concentration of 1.5×10^5 ifu/μl in DMEM. After 24 hours, transduction was supplemented with DMEM 10% FBS.

Phosphatydylserine exposure was determined by flow cytometry after staining with Annexin V, Alexa Fluor 488 conjugate (Thermo Scientific, A13201), and propidium iodide (Sigma, cat. no. P4170). DNA content was determined by propidium iodide staining after cell permeabilization with 70% ethanol.

The clonogenic assay was performed by plating 2000 transduced cells in 10-cm dishes, and colony formation was quantified 12 days later.

Calreticulin exposure was determined by surface staining using rabbit anticalreticulin polyclonal antibody 1:200 (Novus Biologicals, NB300-545) and goat antirabbit conjugate Alexa Fluor 488, 1:500 (Thermo Scientific, A11008) costained with propidium iodide.

Vesicular ATP content [45,46] was assessed by incubating cells with 5 μM quinacrine (Sigma, Q3252-25G) in Krebs-Ringer solution during 30 minutes and then costaining with propidium iodide.

In Vivo Assays

All procedures were evaluated and approved by the Research Ethics Committee as well as the Committee on the Ethical use of Animals, University of Sao Paulo, School of Medicine. Mice were inoculated subcutaneously with 1×10^6 LLC1 tumor cells. When tumors reached a diameter of 3 to 5 mm, adenovector treatment was started. Intratumoral injections were performed every 48 hours for a total of 6 applications. Mice were challenged with an inoculation of 5×10^5 LLC1 cells in the opposite flank of the treated tumor 10 days after the first adenovector application. Cisplatin was intratumorally applied (50 μl at 1 mg ml^{-1}) 24 hours after the fifth adenovector application.

For prophylactic vaccination, 48 hours prior to subcutaneous inoculation, 2×10^6 cells were transduced with adenovectors, and cisplatin (2.5 μM final concentration) was added to transduced cells (12 hours prior subcutaneous inoculations). Mice were challenged with 5×10^5 cells in the opposite flank 7 days after vaccination.

Granulocyte depletion was done by two intraperitoneal applications of GL6-8AC antibody, separated by 4 days, starting with the first adenovector application. Depletion was confirmed by differential blood counts at 24, 48, and 72 hours after IgG injection (data not shown).

Microarray

Tumors were excised 48 hours after the fifth adenovector application; RNA extraction was performed using TRIzol reagent (Thermo Scientific, 15596026) following the manufacturer's instructions. RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, G2939AA). Gene Chip Mouse 1.0 ST (Affimetrix Inc., 901171) was used according to the manufacturer's instructions to determine gene expression. Microarray results were analyzed using TM4 Microarray software suite (Dana-Farber Cancer Institute, USA). Differential gene expression profile was obtained by comparing the p19, IFNβ, and p19 + IFNβ to eGFP-treated tumors using rank product method (false discovery rate < 0.05). Enrichment analysis was performed in DAVID database (EASE score 0.001). Webs of gene interactions were constructed using String database (confidence 0.150).

Quantitative Polymerase Chain Reaction (PCR)

Primers were designed using PrimerBlast (NCBI, USA) and are shown in the 5′ to 3′ orientation: IL1a, Fwd GTCAACTCATTGGCGC TTGA, Rev. GAGAGAGATGGTCAATGGCAGA; Osm, Fwd GCAGAATCAGGCGAACCTCA, Rev. GCTCTCAGGTCAGGTG TGTT; Cd8a, Fwd TTCTGTCGTGCCAGTCCTTC, Rev. GGCCG ACAATCTTCTGGTC; GZMA, Fwd GGGGGCCATCTCTT GCTAC, Rev. AACAACCGTGTCTCCTCCAA; Cd274, Fwd CTC ATGCCAGGCTGCACTT, Rev. ACAAGTCCTTTGGAGCCGTG, Ifng, Fwd CAGCAACAGCAAGGCGAAA, Rev. GTGGACCACTC GGATGAGC; Ccl12, Fwd CACTTCTATGCCTCCTGCTCAT, Rev. CCGGACGTGAATCTTCTGCTT; Ccl17, Fwd CTCTGC TTCTGGGGACTTTTCT, Rev. CAGCACTCTCGGCCTACAT; Cxcl3, Fwd CCACTGCACCCAGACAGAAG, Rev. GGTGAGGGG CTTCCTCCTT; Il1B, Fwd AGTTGACGGACCCCAAAAGA, Rev. GATGTGCTGCTGCGAGATTT; Ccl3, Fwd GCAACCAAGTCTT CTCAGCG, Rev. TGGAATCTTCCGGCTGTAGG). Quantitative PCR was done using Power Sybr Green PCR master mix (ThermoFisher Scientific, 4367659). Expression was calculated by $2^{-\Delta\Delta Ct}$ method, and reference gene was the average expression of β-actin and GAPDH.

Immunohistochemistry

Tumors were excised 72 hours after the fifth adenovector injection, fixed for 4 hours in 4% buffered formamide at room temperature, and then incubated overnight in 30% sucrose at 4°C. Tumors were frozen in Tissue-Tec OCT and stored at −80°C. Slides were prepared by sectioning tumors in a cryostat at 5 μm. Primary antibodies were incubated during 1 hour in PBS 10% FBS solution [Anti CD11b-Alexa 647 Ebioscience, clone M1/70 (1:100); Anti CD11c MBL International, clone 223H7 (1:50); Anti CD86-Alexa 647 Biolegend, clone GL-1 (1:100); Anti CD169 Abcam, 3D6.112 (1:300); Anti F4/80-Alexa 647 Biolegend, clone CI:A3-1 (1:300); Anti cleaved caspase 3 Cell Signaling, clone Asp175 (1:300); Anti Ly6G-PE Biolegend, clone 1A8 (1:100)].

Statistics

Results are expressed as means ± SEM. Statistical significance was assessed by analysis of variance (ANOVA) followed by Tukey posttest or Mann-Whitney *U* test. Distributions in tumor growth were compared by ANOVA followed by Bonferroni posttest. Analyses were performed using GraphPad Prism 6.0.

Results

p19Arf Induces Death in LLC1 Cells

To examine the impact of gene transfer on cell viability, LLC1 cells were transduced with 10^5 ifu/μl of AdRGD-PG-LacZ, AdRGD-PG-p19Arf, AdRGD-PG-IFNβ, or AdRGD-PG-p19Arf and AdRGD-PG-IFNβ. The measurement of phosphatidylserine exposure 72 hours after transduction shows that p19Arf is mainly responsible for induction of cell death (Figure 1*A*); however, association with IFNβ was able to increase this effect. The chemical inhibition of p53-Mdm2 interaction by Nutlin-3 also was able to induce cell death (Figure 1*B*), suggesting a p53-dependent process. A clonogenic assay revealed that combination of p19Arf and IFNβ is able to induce a significant decrease in cell viability even when compared with p19Arf alone (Figure 1*C*). As shown here, whereas p19Arf induces cell death, its association with IFNβ can enhance this effect.

Release of Immunogenic Signals in Response to Combined p19Arf and IFNβ Gene Transfer

Immunogenic cell death, such as induced by anthracyclines, has been shown to be a critical factor for the optimal success of treatment in mouse models. Here we characterized the release of classical ICD signals in response to our gene transfer strategy. As shown in Figure 2*A*, p19Arf gene transfer was able to induce the surface exposure of calreticulin at *premortem* stage (CRT+PI−, 48 hours posttransduction). Moreover, the combination of p19Arf plus IFNβ gene transfer significantly increased CRT+PI− cells when compared with p19Arf gene transfer alone. We also determined that p19Arf is mainly responsible for lysosome loss, revealed by a decrease in quinacrine-labeled cells, indicating ATP release (Figure 2*B*). Strikingly, the combined, but not individual, p19Arf and IFNβ gene transfer resulted in the release of HMGB1 (Figure 2*C*). Taken together, these results indicate that only the combined gene transfer approach promotes the release of all three markers of ICD.

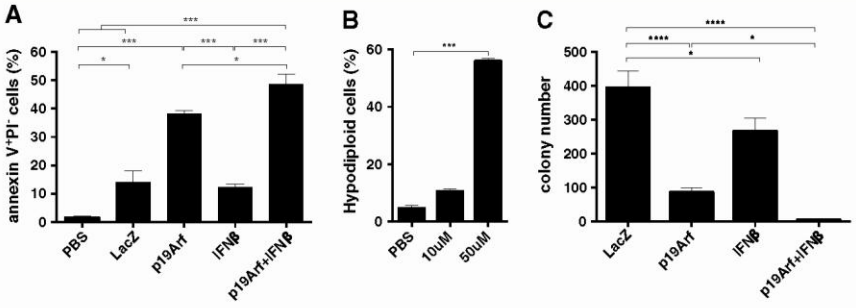

Figure 1. Cell death and viability in LLC1 cells. (A) Cells were transduced with 1.5×10^5 ifu/μl of each adenovector. Annexin V–positive cells were determined by flow cytometry. (B) LLC1 cells were treated with Nutlin-3; hypodiploid cells were determined by flow cytometry after propidium iodide staining. (C) Cell viability was determined in a clonogenic assay. *P < .05, ***P < .001, ****P < .0001 (ANOVA followed by Tukey comparison test in A and B and Mann-Whitney U test in C).

Intratumoral Gene Transfer of IFNβ or Its Combination with p19Arf Significantly Affects Tumor Progression in Immune Competent Host

LLC1 cells were implanted subcutaneously in C57BL/6 or athymic mice; when the tumors attained a diameter of 3 to 5 mm, intratumoral injections of recombinant adenoviruses were performed. The injections were repeated 3, 5, 7, and 9 days after the first application. Treatment with IFNβ, p19Arf, or the combination IFNβ plus p19Arf was able to reduce tumor growth when compared with mice treated with eGFP (Figure 3*A*). However, mice treated with IFNβ showed increased ability to reduce tumor growth when compared with p19Arf alone. Furthermore, intratumoral treatment of athymic mice abrogated the antitumor response when treated with IFNβ or the combination IFNβ plus p19Arf, indicating dependence on the T-cell compartment for controlling tumor growth (Figure 3*B*).

In Situ Gene Therapy with the Combination of p19Arf and IFNβ Induces a Protective Immune Response against Challenge Tumors

To determine if the association of p19Arf and IFNβ gene transfer is able to induce an immune response, the C57BL/6 mice described above were challenged with the implantation of naive LLC1 cells in the flank opposite to the site of the primary, treated tumor. Mice that had the primary tumor treated with the combination p19Arf plus IFNβ showed a significant reduction of tumor growth at the challenge site as compared with animals treated with IFNβ alone (Figure 4*A*). Groups treated with PBS or p19Arf alone were sacrificed early due to primary tumor burden. To confirm this result in a different model, we performed prophylactic vaccination where cells were first transduced *ex vivo* with p19Arf, IFNβ, or both p19Arf and IFNβ. To guarantee that all cells are injected in a similar condition (the same percentage of dead cells), they were also treated with cisplatin, a nonimmunogenic drug [47], 24 hours before vaccination. These cells were injected subcutaneously, and 7 days after vaccinations, mice were submitted to challenge with naive LLC1 cells in the opposite flank. The tendency of mice vaccinated with cells transduced with both p19Arf and IFNβ confirmed our previous observation, indicating an increased immunoprotection (Figure 4*B*) and highlighting a potential benefit of using our combined gene transfer approach.

Differential Gene Expression in Tumors Treated With the Combination of p19Arf and IFNβ

To identify the putative mechanism by which the p19Arf and IFNβ combination impacted immune stimulation, we performed microarray analysis of transcripts expressed *in vivo*. Animals submitted to intratumoral gene transfer were sacrificed 48 hours after the last adenovector injection (day 13), tumors were excised and microarray was performed. Analysis of

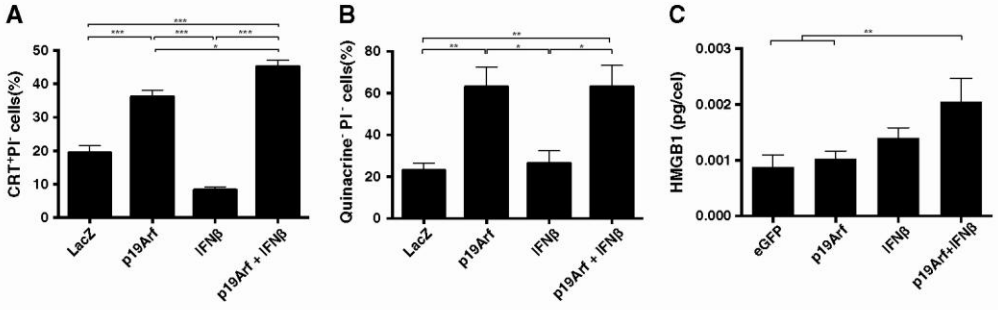

Figure 2. Immunogenic features of p19Arf-induced cell death. (A) Surface calreticulin determined by flow cytometry in live cells 48 hours after adenovector transduction. (B) ATP release was characterized by quinacrine staining 72 hours after gene transfer. (C) HMGB1 release was revealed by ELISA 48 hours posttransduction. *P < .05, **P < .01, ***P < .001 (ANOVA followed by Tukey comparison test, means ± SEM.)

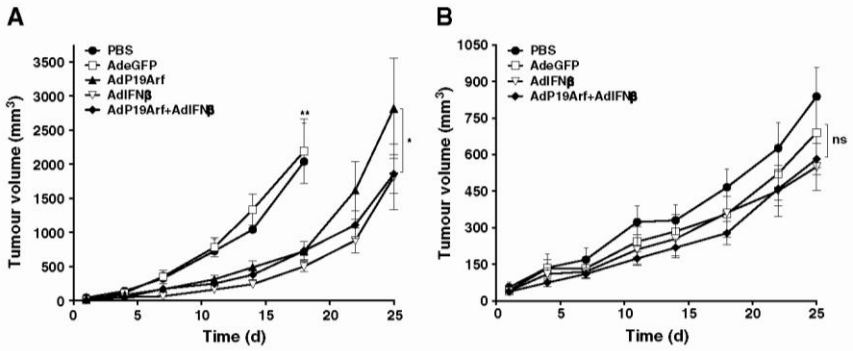

Figure 3. Effect of intratumoral gene transfer on tumor growth. (A) C57BL/6 or (B) athymic mice bearing LLC1 subcutaneous tumors were submitted to intratumoral gene transfer with 10^8 ifu on days 1, 3, 5, 7, 9, and 11. $^{**}P < .01$ compared with eGFP treated mice. $^{*}P < .05$ comparing IFNβ to P19Arf (ANOVA followed by Bonferroni posttest). In A, PBS $n = 6$, eGFP $n = 7$, P19Arf $n = 11$, IFNβ $n = 7$, and P19Arf + IFNβ $n = 7$. In B, $n = 5$ for all groups. Quantitative data are reported as mean ± SEM.

data indicates that combined gene transfer was exclusively responsible for the up- or downregulation of 75 or 87 genes, respectively (Supplemental Figure 1*A*). This set of genes was evaluated *in silico* to verify biological processes that are enriched. Clearly, the differentially expressed genes indicate a strong immune response and chemotaxic enrichment ($P < E^{-6}$) (Supplemental Figure 1*B* and Supplemental Table 1), suggesting involvement of neutrophils and T cells. To identify a subset of genes with pivotal importance in these processes, we constructed a web of genes (Supplemental Figure 2), which allowed us to determine the central proteins. Associating our data with information from the literature, we selected 12 genes for validation by qPCR (CCL2, CCL3, CCL17, IL1β, IL1α, CXCL2, CXCL3, CD8a, GZMA,

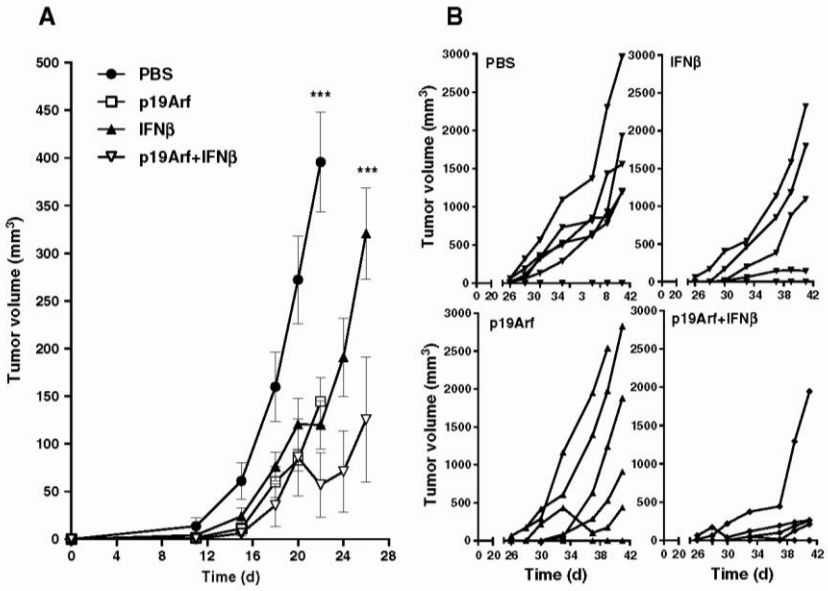

Figure 4. Combination of p19Arf and IFNβ gene transfer increases immunoprotection. (A) Mice whose primary LLC1 tumors were treated with *in situ* gene transfer were submitted to a challenge in the opposite flank; curves represent tumor growth at the challenge site. PBS $n = 5$, eGFP $n = 7$, p19Arf $n = 11$, IFNβ $n = 7$, and p19Arf + IFNβ $n = 7$ (means ± SEM). (B) LLC1 cells were transduced *ex vivo* and used to vaccinate animals, and 7 days after, naive tumor cells were implanted in the opposite flank and their growth was measured ($n = 7$ for all groups, $P < .05$ in PBS versus p19Arf + IFNβ). $^{*}P < .05$, $^{**}P < .01$ compared with PBS (ANOVA followed by Bonferroni posttest).

CD274, OSM, and IFNγ). The quantitative PCR did not confirm the differential expression of CCL2, CCL17, CXCL2, CD8a, GZMA, and IFNγ (data not shown). However, CCL3, CXCL3, IL1α, IL1β, CD274, and OSM showed increase expression only in animals treated with the gene transfer combination (Figure 5).

Impact of p19Arf and IFNβ Gene Transfer on Tumor Infiltrating Myeloid Cells

It is well known that efficient generation of an antitumor immune response is achieved by successfully accomplishing several sequential steps, including antigen presentation, T-cell differentiation/activation, and finally the ability of effector cells to avoid negative regulatory checkpoints [48]. Thus, to determine if the immunostimulatory genes identified in the microarray are reflecting a change in the tumor microenvironment, we investigated if the intratumoral gene transfer impacted infiltration of myeloid cells. Dendritic cells, neutrophils, and macrophages have been described by their ability to present antigens. The quantification of intratumoral $CD11c^+$ $CD86^+$ dendritic cells by immunohistochemistry showed an increase only in IFNβ-treated tumors (Figure 6*A*). Moreover, the $F4/80^+$ $CD169^+$ cells were significantly decreased in the groups treated with IFNβ (Figure 6*B*); curiously, this decrease in the macrophage population seemed to be attributed only to the $CD169^+$ subpopulation because the overall $F4/80^+$ population remained invariable (Supplemental Figure 3*A*). Although the overall myeloid population represented by $CD11b^+$ cells remained invariable (Supplemental Figure 3*B*), the immunohistochemistry revealed an increased number of $CD11b^+$ $Ly6G^+$ cells exclusively in animals treated with the combination of p19Arf and IFNβ (Figure 6*C* and Supplemental Figure S4*A*).

Granulocytic Cell Population Plays a Central Role in Controlling Tumor Growth in Animals Treated with p19Arf and IFNβ

We next determined if the differential infiltration of neutrophils, induced by combined gene transfer, was impacting tumor growth. Animals were treated with intratumoral applications of eGFP or the combination p19Arf plus IFNβ as described previously. The granulocytic cell population in these animals was depleted by intraperitoneal injections of RB6-8C5 antibody. Granulocyte depletion was verified by complete blood count (data not shown). Tumor growth shows that granulocytic population is responsible for the antitumor effects induced by gene transfer (Figure 7). This result does not exclude the involvement of other immune cells, especially T lymphocytes, as indicated by abrogation of antitumor effects in nude mice (Figure 3*B*).

Synergistic Effect of Intratumoral Gene Transfer with Cisplatin Treatment

To approximate common clinical procedures, we associated the gene transfer with a current chemotherapeutic agent, cisplatin (CDDP).

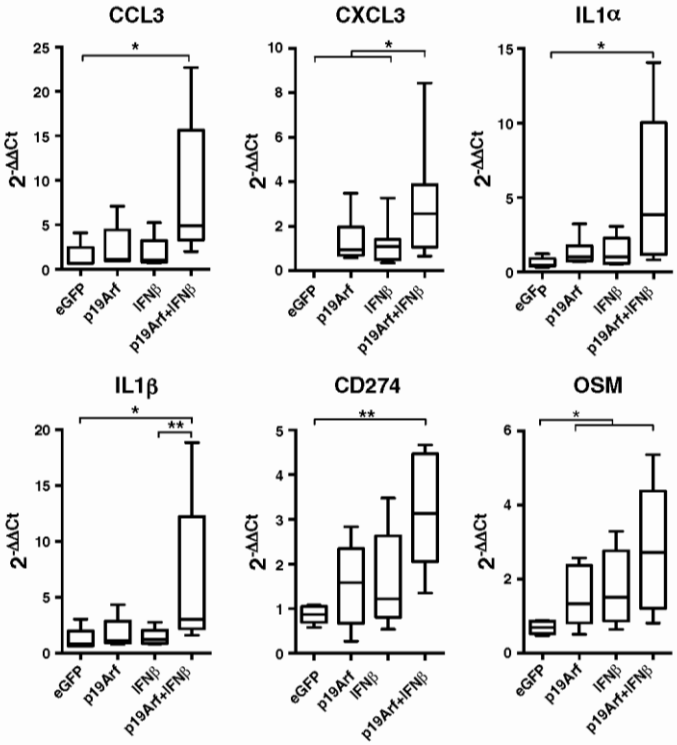

Figure 5. Differential gene expression induced by combined p19Arf and IFNβ gene transfer. Animals were treated as per Figure 3, and tumors were collected 24 hours after the last adenovirus application. CCL3, CXCL3, IL1α, IL1β, CD274, and OSM mRNA expression was confirmed by real-time PCR. *$P < .05$, **$P < .01$ compared with AdeGFP-treated tumors (Mann-Whitney test, means ± SEM).

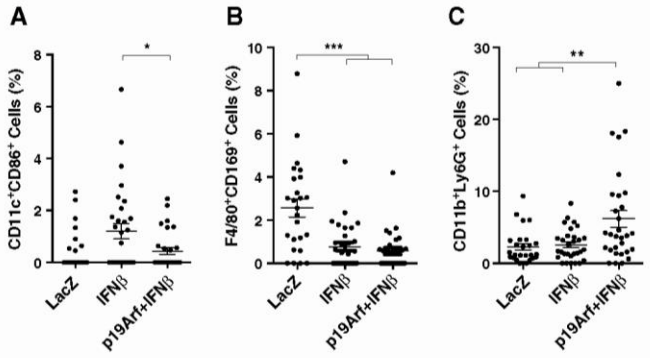

Figure 6. Intratumoral gene transfer induces accumulation of Ly6G+ cells in tumors treated by P19Arf plus IFNβ. Mice were treated with AdLacZ, IFNβ, or p19Arf + IFNβ, and 48 hours after the last (fifth) adenovector injection, tumors were harvested and processed for protein detection by immunohistochemistry to reveal subpopulations of (A) dendritic cells, (B) macrophages, or (C) neutrophils. $*P < .05$, $**P < .01$, $***P < .001$ (ANOVA followed by Tukey comparison test, means ± SEM).

Mice treated with combination gene transfer associated with CDDP showed a decrease in tumor growth compared with either gene transfer or CDDP alone, indicating a strong synergistic effect (Figure 8*A*). Tumors that received CDDP and gene transfer showed an increased number of cells positive for cleaved caspase-3 and an increased number of neutrophils in the tumor bed (Figure 8, *B* and *C* and Supplemental Figure S4, *A* and *B*).

Discussion

In situ vaccination strategies constitute an approach in which an immune response is generated *in vivo* without the previous identification and isolation of a tumor-associated antigen [49]. For decades, *in situ* therapy has been successfully applied to treat bladder cancer, and its mechanisms rely on the modulation of urothelial environment by attracting and activating immune cells to a Th1 response [50]. Currently, several models of intratumoral vaccination, including the application of engineered viruses, have been shown to elicit an effective immune response in different solid tumors [49]. Indeed, with the advent of immunotherapies, new strategies exploiting tumor-associated antigens to generate an antitumor response could constitute a future multimodality therapy [51].

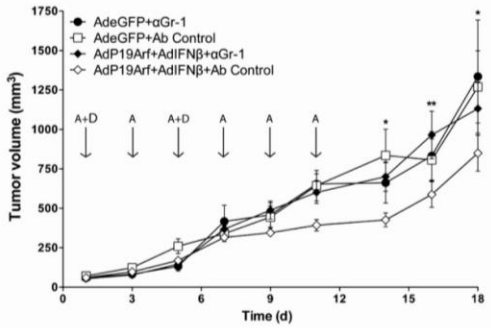

Figure 7. Therapeutic efficacy of p19Arf plus IFNβ gene transfer relies on the granulocytic population. Mice bearing LLC1 tumors were treated by intratumoral applications of eGFP or p19Arf plus IFNβ, combined with either Gr-1–specific or control antibodies. Arrows represent intratumoral application of adenovector ("A") and depeltion ("D") by intraperitoneal antibody injection. $*P < .05$, $**P < .01$ (ANOVA followed by Bonferroni comparison test). Data are reported as mean ± SEM.

Aiming to induce both cell death and antitumor immunity, we have previously generated a p53-responsive adenoviral vector, serotype 5, called AdPG; used this for the transfer of p19Arf or IFNβ to the B16 melanoma cell line; and observed that upon combined transduction, but not individual, a superior induction of cell death was achieved both *in vitro* and *in vivo* [23]. More recently, we employed *ex vivo* transduced melanoma cells in a tumor vaccine model. Although IFNβ alone or in combination with p19Arf resulted in antitumor protection mediated by TH1 CD4+ and CD8+ T cells, only the combination prolonged overall survival, augmented NK cell activity, and reduced tumor progression in a therapeutic vaccine model [41]. Here, we address for the first time the immunogenic properties upon *in situ* gene transfer of p19Arf and IFNβ in a mouse lung carcinoma model. We show that multiple gene transfer (p19Arf and IFNβ) was able to improve the emission of immunogenic signals and modulate myeloid infiltration, and may be a suitable option to increase immunogenicity in the tumor bed.

Our data reveals that inhibition of Mdm2 by either p19Arf or Nutlin-3 yielded similar results, suggesting that p53 is expected to be involved in the cell death mechanism. In addition, by using a p53 responsive promoter, we confirm that endogenous p53 maintains its transcriptional function in this cell line (data not shown). The gene transfer of both p19Arf and IFNβ was able to increase cell death and decrease viability of LLC1 cells. In fact, a few groups have described interaction of p53 and type I interferon pathways [28,52–54], providing additional opportunities for interplay between endogenous p53 and IFNβ for the induction of cell death and immune activation.

ICD has been described as a process in which a stimulus induces the spatiotemporal emission of signals that act by initiating an effective immune response to cellular antigens [55]. Classically, ICD

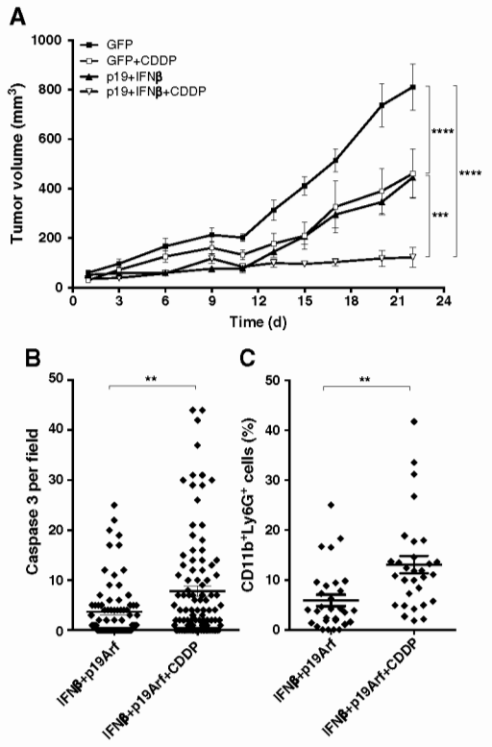

Figure 8. Intratumoral gene transfer of p19Arf plus IFNβ synergizes with current therapies. (A) Mice bearing LLC1 tumors were submitted to intratumoral gene transfer and treated with a single intratumoral application of cisplatin (CDDP) or PBS 48 hours after the last adenovector injection ***$P < .001$, ****$P < .0001$, $n = 5$ per group, (ANOVA followed by Bonferroni comparison test). (B) Quantification of cleaved caspase-3 or (C) accumulation of $Cd11b^+Ly6G^+$ as revealed by immunohistochemistry (**$P < .01$, Mann-Whitney).

induced by certain chemotherapeutic agents, such as anthracyclines, relies on the exposure of calreticulin, release of ATP, HMGB1, and type I IFN [47]. Here, we show that cell death induced by p19Arf is able to induce calreticulin exposure and ATP release; however, only in association with IFNβ was HMGB1 release significantly increased in comparison to controls. In line with these results, *in vivo* experiments demonstrated that association of IFNβ with p19Arf was able to improve the immune response when either *in situ* gene therapy or a prophylactic vaccine was used. Furthermore, IFNβ signaling has recently been shown to be required for the induction of an immune response following treatment with anthracyclines [56], constituting a fourth hallmark of ICD.

Differential gene expression induced by combined gene transfer revealed a cluster of proteins related to the induction of an immune response. Quantitative PCR confirmed the upregulation of Ccl3, Cxcl3, IL1β, IL1α, and OSM, cytokines that are all involved in neutrophil recruitment [57–61]. The individual contribution of each cytokine in our model remains to be elucidated. Because these molecules do not seem to be upregulated LLC1 cells transduced *in vitro* (data not shown), we hypothesize that *in vivo* tumor cell death is responsible for orchestrating neutrophil recruitment. Interestingly and in agreement with our observations, HMGB1 has been associated with recruitment [62] and severity [63,64] of tissue injury by neutrophils during inflammation.

Furthermore, we confirmed by immunohistochemistry an increased population of tumor-associated neutrophils (TANs) in the tumor bed, and this population was shown to be crucial to the antitumor effect induced by p19Arf and IFNβ, as shown by depletion of neutrophils. The contribution of TANs to tumor progression is controversial and seems to be a context-dependent phenomenon. Despite several findings indicating a poor prognostic associated to TANs [65], recent studies have shown that neutrophils exhibit plasticity and can be polarized to an antitumor phenotype [66]. Besides the production of tumoricidal molecules and induction of apoptosis in tumor cells, neutrophils are involved in tumor rejection and immune memory through interactions with $CD8^+$ and $CD4^+$ lymphocytes [67]. The impairment of the antitumor effect observed in athymic mice in our model is consistent with the notion that TANs exert their main activity by this last mechanism. The neutrophil population has been shown to act on several levels of the adaptive response [68], including recruitment [69], antigen presentation [70], and activation of memory cytotoxic T cells [71]. However, in our model, the exact mechanisms involved in T-cell activation/stimulation by neutrophils remain to be investigated in future studies. We cannot rule out the importance of other immune cells in our model, and we have previously shown that NK cells were involved in the antitumor response induced by B16 melanoma cells transduced with p19Arf and IFNβ [41]. Neutrophils have also been shown to exert an antitumor activity by interacting with NK cells; thus, it would be interesting to evaluate whether such collaboration between these cell types occurs in our experimental model [72]. In addition, CD274 upregulation was also observed in our model, and its expression by neutrophils has been described as a mechanism of immunosuppression [73,74], suggesting that association with anti-PD1 therapy could improve the therapeutic effect of p19Arf and IFNβ gene transfer.

In conclusion, we showed that although p19Arf induced cell death, only its association with IFNβ gene transfer was able to fully promote immunogenic hallmarks *in vitro* and immune protection *in vivo*, a finding revealed for the first time in this study. Here we demonstrate a new mechanism in which the response to intratumoral immunostimulation relies on neutrophil recruitment and activity. Finally, the association of our gene transfer approach with CDDP produced a pronounced synergistic effect, indicating that manipulation of the tumor microenvironment can dramatically improve current therapies.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2016.09.011.

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AUTHOR CONTRIBUTIONS

Author contributions: J. P. P. C., R. V. F. M., and B. E. S. conceived and designed the experiments; J. P. P. C., R. V. F. M., A. H., and S. A. performed the experiments; J. P. P. C., R. V. F. M., P. D. V., and B. E. S. analyzed the data; S. A., G. K., D. B. Z., and E. C. S. contributed reagents, materials, and analysis tools; J. P. P. C. and B. E. S. wrote the manuscript.

Specifically, RFVM contributed to the results in Figures 3 a and b; Figure 4 a; Figure 8 a.

6

Reestablishment of p53/Arf and interferon-beta pathways mediated by a novel adenoviral vector potentiates antiviral response and immunogenic cell death

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ARTICLE

Reestablishment of p53/Arf and interferon-β pathways mediated by a novel adenoviral vector potentiates antiviral response and immunogenic cell death

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Late stage melanoma continues to be quite difficult to treat and new therapeutic approaches are needed. Since these tumors often retain wild-type p53 and have a strong immunogenic potential, we developed a gene transfer approach which targets these characteristics. Previously, we have shown that combined gene transfer of p19Arf and interferon-beta (IFNβ) results in higher levels of cell death and superior immune-mediated antitumor protection. However, these experiments were performed using B16 cells (p53wt) with forced expression of the adenovirus receptor and also the mechanism of death was largely unexplored. Here we take advantage of a novel adenoviral vector (AdRGD-PG), presenting an RGD-modified fiber as well as a p53-responsive promoter, in order to investigate further potential benefits and cell death mechanisms involved with the combined transfer of the p19Arf and IFNβ genes to the parental B16 cell line. Simultaneous p19Arf and IFNβ gene transfer is more effective for the induction of cell death than single gene treatment and we revealed that p19Arf can sensitize cells to the bystander effect mediated by secreted IFNβ. Strikingly, the levels of cell death induced upon activating the p53/p19Arf and interferon pathways were higher in the presence of the AdRGD-PG vectors as compared to approaches using pharmacological mimetics and this was accompanied by the upregulation of antiviral response genes. Only combined gene transfer conferred immunogenic cell death revealed by the detection of key markers both *in vitro* and *in vivo*. Finally, whole-genome transcriptome analysis revealed unique expression profiles depending on gene function, including immune activation, response to virus and p53 signaling. In this way, cooperation of p19Arf and IFNβ activates the p53 pathway in the presence of an antiviral response elicited by IFNβ, culminating in immunogenic cell death.

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INTRODUCTION

Malignant melanoma is a type of cancer with high death rates due, in part, to the lack of efficient treatments once metastases have formed.[1] The tumor suppressor protein p53 is found in its wild-type form in 90% of melanoma cases, though other components of this pathway may be altered. Tolerance to p53wt during melanomagenesis can be achieved through the overexpression of its negative regulator HDM2, found in 56% cases,[2,3] and loss of its functional partner on the CDKN2A locus (where p14ARF resides), which occurs in some 50% of primary melanomas.[4] As p53wt is maintained at such a high frequency in melanoma, we and others propose that it may be recruited to participate in the therapeutic approach.[5–7]

Previously, we showed that introduction of the p53 functional partner, p19Arf (p19Arf in mice, p14ARF in humans) into B16 melanoma cells restored the p53 pathway and promoted cell death *in vitro* and *in vivo*.[8] However, the impact of apoptotic cell death may be limited and is not likely to actively promote an antitumor immune response. Therefore, we sought to develop a cancer gene therapy approach that would induce cell death by a mechanism that would have wide-spread anti-cancer effects that reach beyond the treated cell.

Interferon-β (IFNβ) is a pleiotropic cytokine with cytostatic, anti-angiogenic, pro-apoptotic and immunomodulatory activities[9] and it interacts with the p53 pathway. For example, IFNβ and p53 cooperate in antiviral defense[10–12] while other works have implied that p14ARF is critical for induction of apoptosis in response to type I interferon.[13] Together, these data suggest a promising benefit of using p19Arf in combination with IFNβ for the induction of cell death.

Even high levels of cell death may lack the signaling necessary to promote an adaptive immune response that could then continue tumor killing at the site gene therapy and, possibly, at distant foci. Such signaling is associated with immunogenic cell death (ICD), including the exposure of calreticulin (CRT) and the release of ATP and HMGB1 by the dying cell, serving to stimulate the maturation of dendritic cells and elicit an efficient antitumor response.[14] Many cellular pathways contribute to ICD, including endoplasmic reticulum stress, necroptosis and components of

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autophagy.[15–17] The participation of type I interferon is also a key component of ICD[18] and is a requirement for immune rejection of tumors,[19] but the use of gene transfer for this purpose has not been widely examined.

Indeed, in our first attempt to explore the p19Arf and IFNβ pathways, it was revealed that the combined delivery of this genes, but not individual applications, leads to an increase in B16 cell death *in vitro* and *in vivo* in a gene therapy model.[20] Also, melanoma cells were transduced *ex vivo* and these dying cells were applied as an immunogen, conferring protection in a prophylactic vaccine model as well as tumor reduction in a therapeutic vaccine assay,[21] but the mechanism of cell death was not explored in detail and thus the reason why the p19Arf+IFNβ-treated cells respond with a superior immune stimulus remained unclear. In these previous works, we used adenoviral vectors with the wild-type fiber to transduce B16 cells with forced expression of the coxsackievirus and adenovirus receptor (CAR).[20,21] Forced expression of CAR may alter cellular phenotype[22] and broad application of our gene transfer approach was limited by the need for the CAR-mediated adenoviral transduction of target cells. Here we present for the first time the effects of a novel adenoviral vector with an RGD-modified knob protein for increased viral tropism. In this system, the p19Arf and IFNβ transgene expression is controlled by a p53-responsive promoter, which was developed in our laboratory and confers higher-gene expression and superior antitumor activity when compared to the CMV promoter.[23,24]

Taking advantage of this novel delivery system, we revealed (i) the bystander effect of IFNβ produced by transduced cells on the non-transduced cells, (ii) the mechanisms of cell death unleashed by p19Arf and IFNβ gene transfer, involving the release of ICD molecules by dying cells and (iii) that the activation of p53 pathway, in the presence of an endogenous antiviral response elicited by IFNβ, culminates in massive cell death. Together, these results provide new insights as to why combined, but not individual, transfer of p19Arf and IFNβ leads to cell death associated with superior antitumor immune protection.

RESULTS

Combined p19Arf and IFNβ gene transfer enhances cell death *in vitro* and arrests tumor growth *in vivo* involving activation of the p53 pathway

The AdRGD-PG vectors were established by the introduction of the p53-responsive promoter, called PG, resulting in a vector platform where transgene expression would be controlled by p53. The set of AdRGD-PG vectors constructed includes three monocistronic vectors, containing the eGFP, p19Arf or murine IFNβ cDNAs and a bicistronic vector containing the IRES element between the p19Arf and IFNβ genes (Supplementary Figure S1). These RGD-containing vectors can successfully transduce CAR-negative B16 cells (Supplementary Figure S2A) and express the reporter gene eGFP in a p53-responsive manner (Supplementary Figures S2B and C). The expression of p19Arf and IFNβ by the mono and bicistronic vectors was also confirmed (Supplementary Figures S3 and S4).

Next, we observed the effects of gene transfer on B16 cells. Either p19Arf or IFNβ reduced proliferation (Figure 1a), yet mitochondrial activity was reduced to a greater degree when the genes were combined (Figure 1b). We also observed that only combined treatment with p19Arf+IFNβ resulted in altered cell cycle distribution (Supplementary Figure S5) and dramatically increased hypodiploid cells when compared to individual gene transfer (Figure 1c). Moreover, qPCR analysis of *Trp53* and its target genes (*p21^{Waf1}*, *Puma* and *Phlda3*) revealed activation of this pathway especially upon combined treatment *in vitro* (Supplementary Figure S6).

The *in vivo* effect of combined p19Arf and IFNβ gene transfer was verified in B16 tumors after *in situ* injections of AdRGD-PG vectors. Animals were accompanied for tumor growth or tumors were collected for evaluation of gene expression. As expected, p19Arf+IFNβ treatment *in vivo* significantly increased transcript levels for *Trp53*, *p21^{Waf1}*, *Puma* and *Phlda3* (Figure 1d) and significantly reduced tumor progression (Figure 1e). Note that even though IFNβ treatment reduced tumor growth, lower or no increase in gene expression was observed in this condition as compared to p19Arf or controls. As shown here, the new AdRGD-PG vectors present a technological advantage, the treatment of CAR-negative cells.

IFNβ bystander effect and its enhancement in cells harboring p19Arf

Although IFNβ is a secreted protein, we hypothesize that even a small percentage of cells receiving AdRGD-PG-IFNβ could have an impact on non-transduced neighboring cells. As seen in Figure 2a, when 10% of cells were transduced with AdRGD-PG-IFNβ, the accumulation of hypodiploid cells was similar to that observed when 100% of cells were transduced with the IFNβ vector. When we mixed cells transduced with the p19Arf adenoviral vector with cells transduced with the IFNβ or the bicistronic vectors (9 : 1 proportion), the number of hypodiploid cells increased, suggesting that p19Arf can sensitize the cells and augment the bystander effect mediated by IFNβ.

The cellular response to IFNβ was confirmed using a stably modified B16 cell line where expression of both GFP and luciferase reporter genes is under control of the interferon-stimulated response element, ISRE (here after called B16ISRE-GFP-Luc). When transduced and co-cultivated as described above, we observed that 10% of cells transduced with AdRGD-PG-IFNβ were sufficient to induce reporter gene expression in more than 40% of the population, essentially the same as seen when transducing 100% of the cells with AdRGD-PG-IFNβ (Figure 2b). When p19Arf-transduced B16ISRE-GFP-Luc cells were co-cultivated with IFNβ-producing cells, the induction of reporter gene activity was reduced, presumably due to cell death. No significant induction of ISRE was caused by transduction with AdRGD-PG-p19Arf. Treatment with the GFP adenoviral vector was used as a control to quantify the transduction efficiency. Together, these results indicate a strong bystander effect mediated by IFNβ that is further enhanced in the presence of p19Arf.

The bystander effect of IFNβ would be particularly interesting *in vivo* since we do not expect that all tumor cells are transduced upon gene therapy. To verify this, B16ISRE-GFP-Luc cells were inoculated in C57BL/6 mice and tumors were treated *in situ* with AdRGD-PG-eGFP or AdRGD-PG-IFNβ vectors. By assaying GFP-positive cells 24 h later, we verified that AdRGD-PG-eGFP transduced only 45% of tumor cells, but the IFNβ bystander effect is extended remarkably to 70% of cells (Figure 2c). Luciferase activity was also measured revealing induction of ISRE only when tumors were treated with the AdRGD-PG-IFNβ vector (Figure 2d). These data demonstrate that the number of cells affected by IFNβ *in vivo* can be much greater than the number of cells transduced by the adenoviral vector, indicating that the bystander effect broadens the impact of gene therapy at the site of treatment.

p19Arf and IFNβ gene transfer elicits an antiviral response in B16 cells

To better characterize the induction of cell death upon simultaneous stimulation of the p19Arf and IFNβ pathways, we compared pharmacological approaches with gene transfer of p19Arf or IFNβ. We used recombinant IFNβ (IFN-R) protein instead of the AdRGD-PG-IFNβ vector and nutlin-3, a compound which inhibits the interaction between MDM2 and p53,[25] instead of the

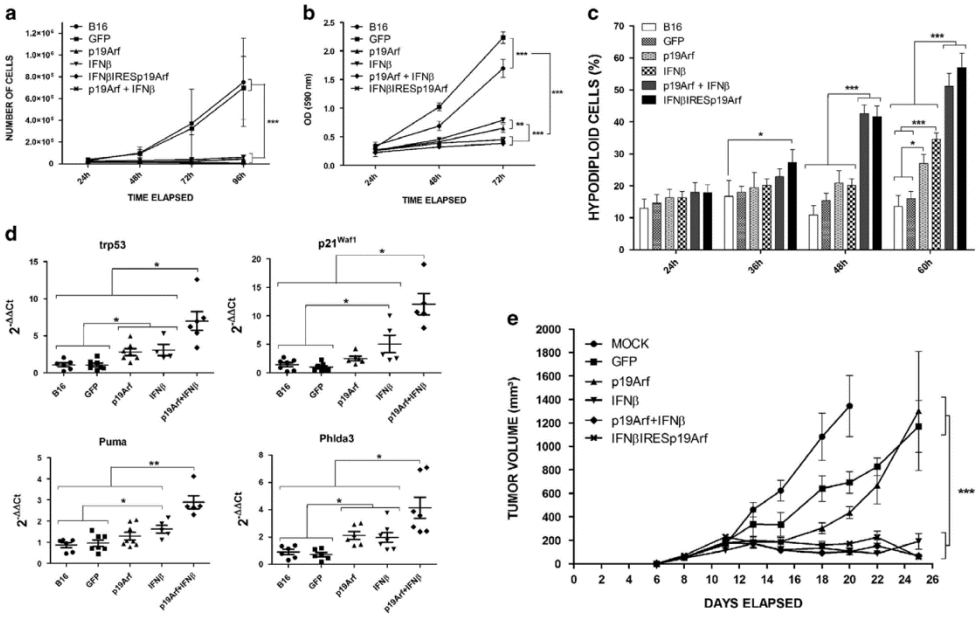

Figure 1. Gene transfer of p19Arf and IFNβ induces accumulation of hypodiploid cells and p53 pathway genes. B16 cells were transduced with the AdRGD-PG vector and (**a**) replated for assessment of growth potential measured by counting viable cells or (**b**) replated in 96-well plates and quadruplicate samples were stained for MTT assay. (**c**) Cells were also analyzed by flow cytometry for evaluation of hypodiploid cells. Results from each graph in **a**, **b** and **c** represent the average and s.d. among at least duplicate samples from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, two-way analysis of variance, followed by Bonferroni. (**d**) Mice were inoculated with B16 cells and treated with the AdRGD vectors. Tumors were collected for RT-qPCR analysis of trp53 pathway genes (*Trp53*, *p21^{Waf1}*, *Puma* and *Phlda3*). β-actin and/or glyceraldehyde 3-phosphate dehydrogenase were used as the reference genes. Data represent the average and s.d. from duplicated PCR reactions derived from 7–10 animals per group. $^{*}P < 0.05$ and $^{**}P < 0.01$, one-way analysis of variance, followed by Kruskal–Wallis. (**e**) In parallel, mice were maintained for tumor size evaluation. Results represent the average and s.d. of tumor volumes (mm^3). $N = 6$ for all groups except Mock and GFP, where $N = 4$. $^{***}P < 0.001$, two-way analysis of variance, followed by Bonferroni.

AdRGD-PG-p19Arf vector. As shown in Figure 3a, the accumulation of hypodiploid cells upon treatment with IFN-R+nutlin-3 (20–30%) was far inferior to that seen with p19Arf and IFNβ gene transfer (60–65%). This result implies that our gene transfer approach is superior to pharmacologic treatment.

The combination of nutlin-3 with AdRGD-PG-IFNβ provided significant levels of cell death (Figure 3b), suggesting that the critical role of p19Arf gene transfer is to inhibit p53–MDM2 interaction and that other functions of p19Arf are not essential here. Similarly, as shown in Figure 3c, treatment with IFN-R resulted in significant accumulation of hypodiploid cells only when combined with AdRGD-PG-p19Arf.

To confirm this result, we used the double-stranded RNA analog Poly (I:C), which is a toll-like receptor-3 agonist shown to induce IFNβ production by B16 cells.[26] As expected, Poly (I:C) treatment promoted IFNβ production (Figure 3d) and, when associated with AdRGD-PG-p19Arf, induced similar levels of hypodiploid cells compared to combined gene transfer (Figure 3e).

Strikingly, combined Poly (I:C)+nutlin-3 treatment did not affect tumor cells, but did only in the presence of the AdRGD-PG-eGFP vector (Figure 3f). Cell death was induced in a multiplicity of infection (MOI) responsive manner, reaching a level comparable to that seen with AdRGD-PG-IFNβ+AdRGD-PG-p19Arf treatment. Together, these data suggest that the gene transfer approach results in higher levels of cell death as compared to the use of IFN-R or Poly (I:C) together with nutlin-3 and that the success of the pharmacologic approach requires transduction with an adenoviral vector during simultaneous stimulation of both p53/MDM2/Arf and IFNβ pathways.

Since the presence of the adenoviral vector is required, we evaluated the expression of genes related to antiviral response in B16 cells treated with p19Arf and IFNβ gene transfer. We show that the transcripts of *Dram1*, a p53-responsive regulator of autophagy[27] that can be induced by viral infection,[28,29] *Chop*, an unfolded protein response-activated transcription factor involved in antiviral response[30] and *Nlrc5*, a classical antiviral response gene,[31,32] were all induced to higher levels in the presence of p19Arf+IFNβ (Figure 3g). In addition, *Isg15* mRNA, another mediator of the antiviral response[33] and a p53 target gene,[34] was induced by IFNβ gene transfer alone or in combination with p19Arf (Figure 3g). Together, these data imply that an endogenous antiviral response provoked upon adenovirus transduction in the presence of p19Arf and IFNβ activity is a key component of our gene therapy approach.

In order to corroborate the antiviral response *in vivo*, tumors treated with *in situ* gene therapy were analyzed and an increase in *Isg15*, *Nlrc5*, *Dram1* and *Chop* gene expression was detected in cells treated with p19Arf or IFNβ (Supplementary Figure S7A). Here the occurrence of autophagy *in vivo* was also verified by LC3β perinuclear staining only in cells treated with p19Arf+IFNβ (Supplementary Figures S7B and C). These data show that the cell death mechanisms observed *in vitro* were preserved *in vivo*,

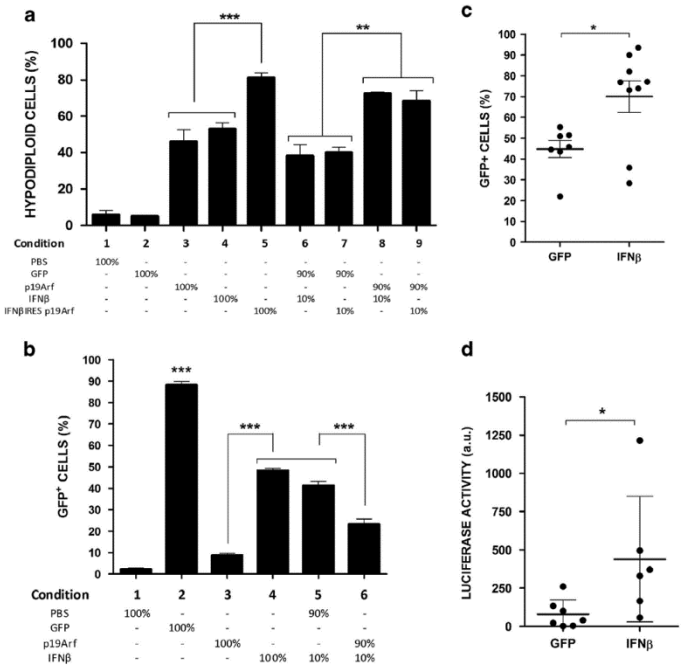

Figure 2. p19Arf can sensitize cells to IFNβ bystander effect *in vitro* and *in vivo*. Cells were transduced with the indicated AdRGD-PG vectors and later mixed in the indicated proportions in six-well plates. (**a**) B16 cells were collected 72 h post transduction and accumulation of hypodiploid cells was revealed by flow cytometry. (**b**) B16ISRE-GFP-Luc cells were collected 48 h post transduction and the percentage of eGFP-positive cells was measured by flow cytometry. Results from each graph represent the average and s.d. from three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way analysis of variance, followed by Tukey's multiple comparison. For detection of the IFNβ bystander effect *in vivo*, mice were inoculated with B16ISRE-GFP-Luc cells and treated with the AdRGD vectors as described in Figure 1. Tumors were collected 1 day after the last injection. (**c**) Detection of GFP indicates level of B16 transduction by AdRGD-PG-eGFP or bystander effect of AdRGD-PG-IFNβ vectors. (**d**) Detection of luciferase activity reveals lack of bystander effect by AdRGD-PG-eGFP, but influence of AdRGD-PG-IFNβ. Results represent the average and s.d. from tumors of at least six animals in each group. $*P < 0.05$, *t*-test.

including activities seen only upon combined p19Arf+IFNβ gene transfer.

p19Arf and IFNβ gene transfer is associated with ICD

Next, we further explored the cell death mechanism elicited by our gene therapy approach. Even though treatment with p19Arf +IFNβ conferred an increase in Annexin V-positive cells (Figure 4a), the expression of Bax (Figure 4b) and the activity of caspase-3 (Figure 4c), seen by a luciferase reporter vector,[35] were surprisingly lower when compared to p19Arf treatment. In this regard, treatment of B16 cells with the pan-caspase inhibitor Z-VAD-FMK did not reduce cell death triggered by p19Arf+IFNβ (Figure 4d). Surprisingly, Z-VAD-FMK treatment prior to transduction with p19Arf or IFNβ increased the hypodiploid population (Figure 4d), pointing to a caspase-independent mechanism of cell death.

Necroptosis was originally identified as an alternative cell death program activated when caspase was blocked and is now also recognized as cellular defense mechanism against infection, including dsDNA viruses.[36,37] Expression of both RIP3 (Figure 5a), the key mediator of necroptosis[38] and the TNF receptor (*Tnfrsf1a*, Figure 5b), an activator of the necrosome complex,[38] were increased only in cells treated with p19Arf+IFNβ.

Recently, necroptotic cells have been shown to undergo ICD upon chemotherapy.[39] Similarly, as observed in Figure 5c, only combined gene transfer resulted in the increase of all three markers of ICD, CRT exposure, secretion of ATP and release of HMGB1, as compared to the other conditions. Taken together, these data indicate that p19Arf+IFNβ treatment induces a cell death mechanism with features of necroptosis and culminates in ICD.

Molecular pathways and cellular functions associated with p19Arf and IFNβ treatment in B16 cells

To further elucidate the pathways that underlie cell death induced by p19Arf and IFNβ treatment, whole-genome transcriptome analysis was performed. The unsupervised hierarchical clustering of samples was distinct for each of the conditions (GFP, p19Arf, IFNβ and p19Arf+IFNβ; Supplementary Figure S8) and K-means analysis revealed that four gene clusters and pathways are modulated according to the different treatments (Figure 6a). Genes involved in immune response, response to virus and antigen processing were strongly upregulated with IFNβ and p19Arf+IFNβ treatments (clusters 1 and 4 – additional file chart_cluster 1 and 4), evidenced by the genes *Trim30*, *Ifi44*, *Usp18*, *Isg15*, *Cxcl9*, *Il18*, *Tlr3* and *Nlrc5*, for example. On the other

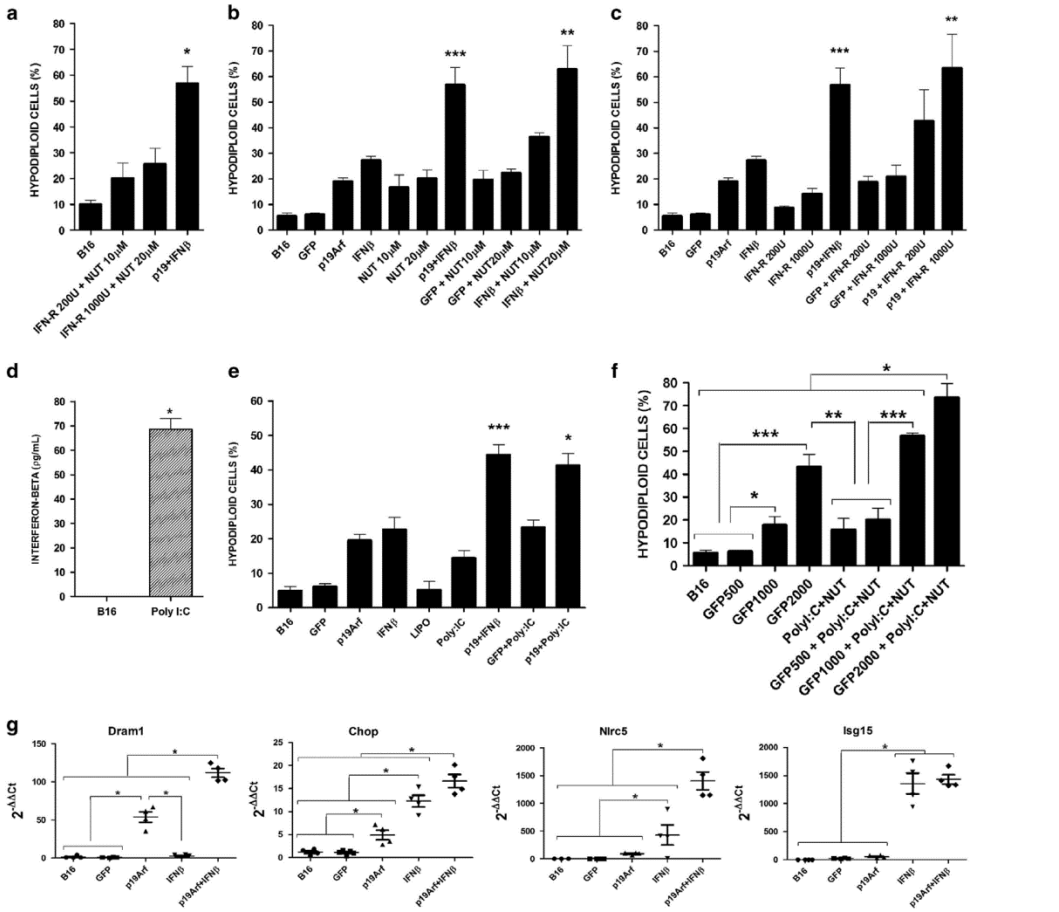

Figure 3. Cell death induced by p19Arf+IFNβ treatment depends on transduction by adenoviral vectors. B16 cells were transduced with the AdRGD-PG vectors (MOI 500 or as stated in figure), treated with nutlin-3, IFN-R or transfected with Poly (I:C) and, 72 h after, collected for flow cytometric analysis of hypodiploid cells. (**a**) Combined pharmacologic treatment. (**b**) Use of nutlin-3 instead of the p19Arf vector. (**c**) Use of IFN-R instead of the IFNβ vector. (**d**) Production of IFNβ upon transfection with Poly (I:C) (0.2 μg/ml) ($^*P < 0.05$, *t*-test). (**e**) Use of Poly (I:C) (0.2 μg/ml) in place of IFN-R. (**f**) Contribution of vector to cellular response upon pharmacological treatment (Poly (I:C) 0.2 μg/ml+nutlin-3 10 μM). Results from each graph represent the average and s.d. from at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way analysis of variance, followed by Tukey's multiple comparison. (**g**) RNA was extracted from transduced cells (36 h) and RT-qPCR analysis of *Dram*, *Chop*, *Nlrc5* and *Isg15* was performed. β-Actin and/or glyceraldehyde 3-phosphate dehydrogenase were used as the reference genes. Data represent the average and s.d. from duplicate PCR reactions derived from five independent biological experiments. $^*P < 0.05$ one-way analysis of variance, followed by Kruskal–Wallis.

hand, genes involved in the p53 signaling pathway and apoptosis are upregulated by p19Arf and p19Arf+IFNβ treatments (cluster 2 – additional file chart_cluster 2), enriched by *Fas*, *Gadd45b*, *Trp53inp1* and *Casp3* genes. Molecular pathways involved in cell cycle control indicate that this function is being strongly affected by p19Arf, IFNβ or p19Arf+IFNβ treatments (cluster 3 – additional file chart_cluster 3), as indicated by the downregulation of *Ccna2*, *Ccnb1*, *Aurka* and *Aurkb* genes, among others (Figure 6b).

These results provide a molecular framework for the induction of cell death triggered by the reestablishment of the apoptotic p53/Arf pathway in the presence of an IFNβ antiviral response, demonstrating that cell death is a result of cooperative functions between these two pathways.

Based on the data presented here and in our previous studies, we propose the following mechanism (Figure 7). First, reintroduction of p19Arf blocks MDM2 function and frees p53 to activate its pro-apoptotic pathway, as demonstrated by upregulation of p53 pathway genes, increase in caspase-3 activity and Bax expression.[20] However, the cell death stimulus (blue arrow) of the p19Arf treatment is not strong enough to provoke high levels of cell death. At the same time, upon treatment with IFNβ, an antiviral defense mechanism is activated, evidenced by the

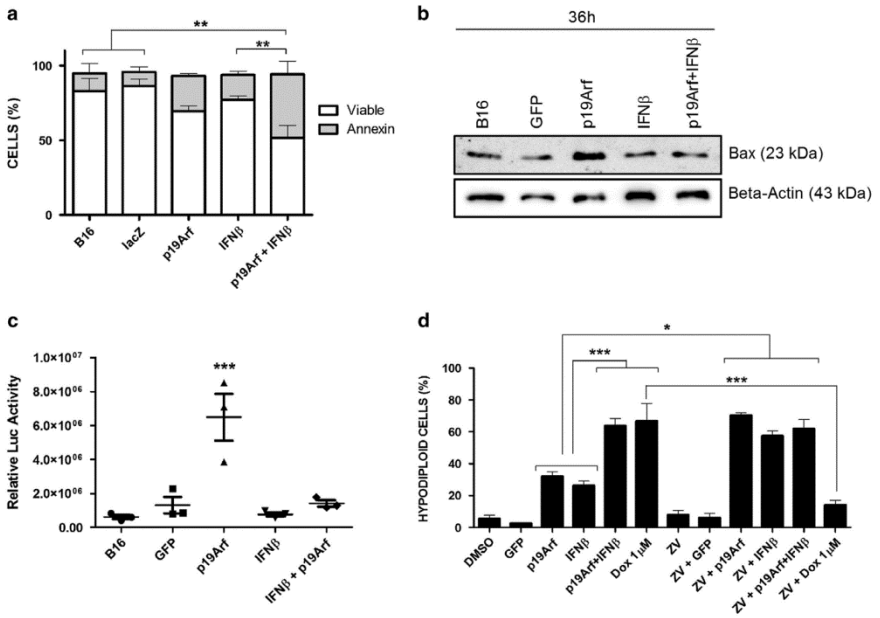

Figure 4. p19Arf gene transfer induces apoptosis but p19Arf+IFNβ induces caspase-independent cell death. (**a**) Analysis of phosphatidylserine exposure by AnnexinV-PI double staining 72 h post transduction. Data represent the average and s.d. from three independent biological experiments $^{**}P < 0.01$ two-way analysis of variance, followed by Bonferroni (**b**) Bax expression detected by western blot analysis of protein extracts from B16 cells 36 h post transduction. (**c**) Caspase-3 activity as measured by luciferase reporter gene activity 36 h after transduction. (**d**) Treatment of cells with Z-VAD-FMK prior to treatment with adenoviral vectors indicates that p19Arf+IFNβ induces cell death independent of caspase activity. Data (**c**, **d**) represent the average and s.d. from at least three independent biological experiments $^{*}P < 0.05$, $^{***}P < 0.001$, one-way analysis of variance, followed by Tukey's multiple comparison.

microarray analysis, which allows B16 cells to detect adenovirus components, most likely dsDNA in the cytoplasm through the DAI dsDNA sensor. In this regard, without the presence of the adenovirus, the activation of p19Arf and IFNβ pathways is not enough to induce death. However, when the p53/Arf and antiviral IFNβ pathways are combined, a cooperative context is achieved and the stimulus is strong enough to provoke massive cell death. This cellular death process displays features of necroptosis, suggested by RIP3 expression, upregulation of the TNF receptor and the absence of caspase-3 activity. On the other hand, in the extracellular environment, ICD markers (ATP, CRT and HMGB1) are released along with the secretion of IFNβ to promote an antitumor immune response, resulting in the activation of NK cells, CD4+ and CD8+ T lymphocytes and immune protection to the host, as seen in our previous work.[21] As shown here, IFNβ can also affect non-transduced cells trough a bystander effect and possibly, due to its established anti-angiogenic activity, can affect the surrounding blood vessels as well.

DISCUSSION

The work presented here elucidated several molecular and cellular mechanisms induced by the adenovirus-mediated gene transfer of p19Arf together with IFNβ that culminates in ICD of B16 melanoma cells.

We describe the utilization of a vector platform modified to include the RGD tripeptide in its fiber, allowing for the efficient transduction in a wide range of target cells without dependence on the Ad5 receptor, CAR. In addition, a bicistronic vector was constructed which contains the combination of therapeutic genes, ensuring the transfer of both factors to the target cells at the same time.

The influence of IFNβ bystander effect on non-transduced cells was confirmed *in vivo*, yet the presence of p19Arf-sensitized B16 cells to IFNβ *in vitro*, showing that the antitumor effects of our treatment extend beyond the transduced cells, possibly affecting a variety of cell types in the tumor microenvironment. To the best of our knowledge, this is the first report showing the extent of the IFNβ bystander effect upon *in situ* gene therapy mediated by an adenoviral vector.

In situ application of our p19Arf+IFNβ therapy effectively inhibited tumor growth correlating with the upregulation of *Trp53*, *p21^{Waf1}*, *Puma* and *Phlda3* mRNA levels. Even though IFNβ by itself could also inhibit tumor growth, our previous observations indicate that only p19Arf+IFNβ treatment confers superior immune stimulation involving the NK cells, CD8+ and CD4+ T cells.[21] Here we have evidenced the release all three classic markers of ICD (CRT, ATP and HMGB1), providing a likely mechanism for the results seen in our previous study.

Interactions of p53/Arf and IFNβ pathways have been shown to play a role in controlling virus infection including through the induction of cell death.[11,12] Here we have shown that the presence of the adenoviral vector and the activation of the p53/Arf and IFNβ pathways are critical for the induction of high levels of cell death.

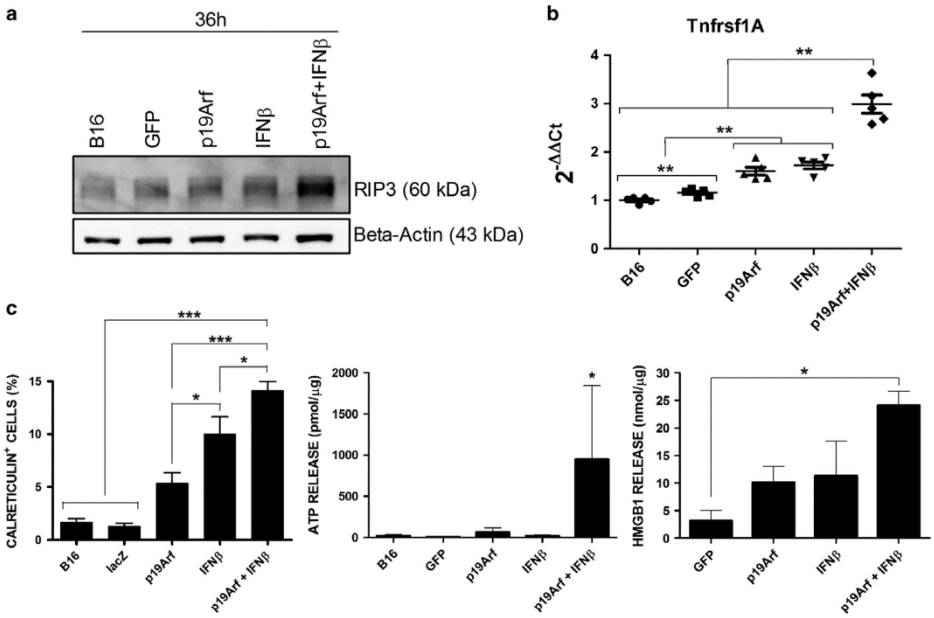

Figure 5. Gene transfer of p19Arf+IFNβ induces features of necroptosis and ICD. (**a**) Protein levels of RIP3 were evaluated from B16 extracts and were increased upon p19Arf+IFNβ treatment. (**b**) Total RNA extraction and RT-qPCR analysis of cells treated with AdRGD-PG vectors show an increase in TNFRSF1A (TNFR) expression after p19Arf+IFNβ treatment. β-Actin and/or glyceraldehyde 3-phosphate dehydrogenase were used as the reference genes. Data represent the average and s.d. from duplicated PCR reactions derived from five independent biological experiments. $^{**}P < 0.01$ one-way analysis of variance, followed by Kruskal–Wallis. (**c**) Cells were collected for flow cytometric analysis of CRT exposure and supernatant was collected for measure of secreted ATP or HMGB1 release by B16 cells. Graphs represent the average and s.d. from at least three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$, one-way analysis of variance, followed by Tukey's multiple comparison.

Indeed, IFNβ only induced significant cell death with the concomitant inhibition of MDM2 and in the presence of the viral vector. These observations were further supported by the induction of the *Isg15*, *Nlrc5*, *Dram* and *Chop* genes, involved in antiviral response.[33,40,41] Moreover, transcriptome analysis of genes activated by IFNβ also revealed a clear antiviral molecular signature, thus providing support to our proposed model.

Innate antiviral responses to adenovirus are initiated upon the interaction of the RGD motif with αv integrins. After being internalized, adenoviral dsDNA can be sensed within the endosome via TLR9 and in the cytoplasm by the DNA-dependent activator of IFN-regulatory factors (DAI) and by NOD-like receptors (NLRs).[42] Interestingly, DAI induces an IFN response[43,44] and is able to sensitize cells to virus-induced necroptosis by directly activating RIP3 without the involvement of RIPK1.[37] Given our observation that RIP3 was induced by p19Arf +IFNβ gene therapy, an ideal context for the induction of necroptosis seems to be met when using our adenoviral vector for the transfer of these genes. Indeed, a recent report in the literature showed that RIP3 is critical for the induction of ICD, since dying cancer cells deficient in RIP3 were not able to induce an immune response in mice.[39]

In conclusion, using the novel AdRGD-PG vectors, the combined p19Arf and IFNβ gene therapy approach induces high levels of cell death, summons the immune system to the battle and, as a result, is predicted to have a wide-spread impact on tumor inhibition. The mechanism of cell death includes reactivation of the p53/Arf apoptotic pathway, induction of innate antiviral response and ICD with involvement of necroptosis. While much development is required, we propose that the combined gene transfer approach of IFNβ with p14ARF will bring superior clinical benefit not seen with IFNβ biochemotherapy or gene therapy strategies that are only focused in killing tumor cells without activating an immune response.

MATERIALS AND METHODS

Cell culture and lines

The adenovirus-transformed, human embryonic kidney cell line 293A (HEK293A, Invitrogen, Carlsbad, CA, USA) was maintained in DMEM (Invitrogen) supplemented with 10% bovine calf serum (HyClone, Logan, UT, USA), 100 μg/ml gentamicin, 50 μg/ml ampicillin and 2.5 μg/ml fungizone, at 37 °C, in a humidified atmosphere of 5% CO_2. The mouse melanoma cell line B16F10 (B16, ATCC CRL-6475, confirmed presence of mouse short-tandem repeats and the MART1, S100A1, SOX10 and TYR markers of melanoma, data not shown) was cultured as above, except using Roswell Park Memorial Institute medium (Invitrogen). B16mCAR cells have been described previously.[20] The lentiviral caspase-3 reporter vector, which encodes a constitutively expressed luciferase-GFP protein separated from a polyubiquitin domain via a caspase-3 cleavage site,[35] was obtained from Chuan-Yuan Li (Department of Radiation Oncology, University of Colorado School of Medicine, Aurora, CO, USA). The pGreenFire1-ISRE reporter construct, a lentiviral vector which expresses both GFP and luciferase in response to type I IFN signaling, was obtained commercially (System Biosciences, Mountain View, CA, USA). These vectors were used to transduce B16 cells. B16 cells transduced with caspase-3 reporter vector were selected for puromycin resistance (0.5 μg/ml) and B16 cells

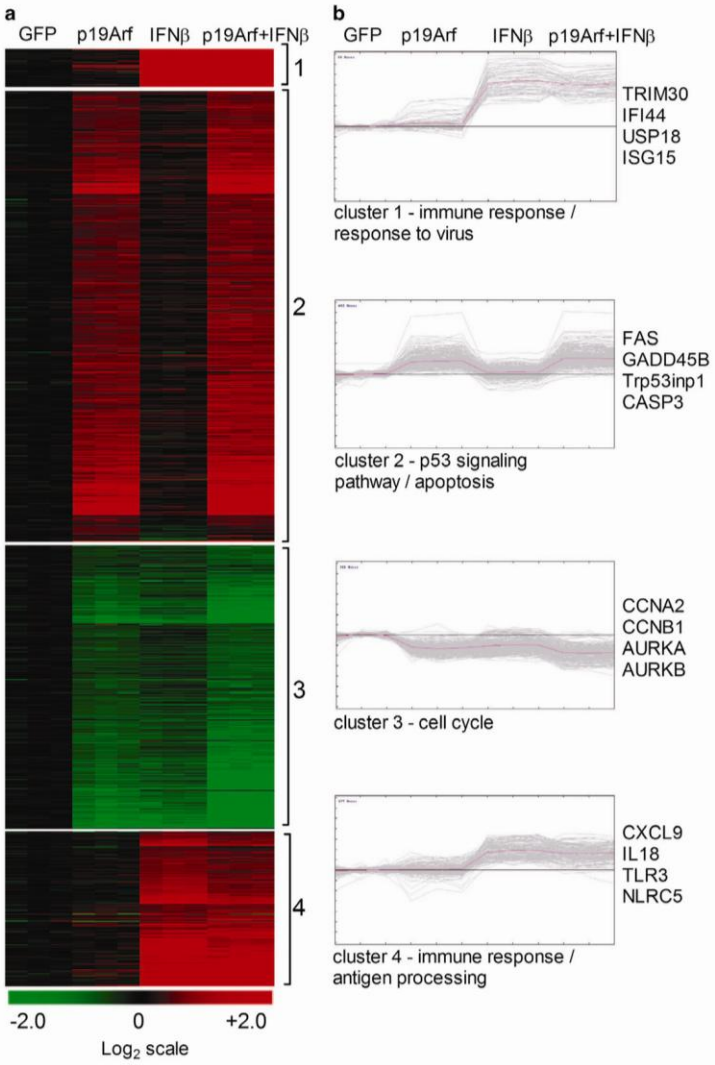

Figure 6. Genome-wide transcriptome analysis of p19Arf and IFNβ-treated cells. (**a**) K-means clustering shows heatmap of transcript levels for the four clusters (log2-tranformed expression intensities); (**b**) Selected K-means clusters showing genes involved in different functions. Cluster 1 represents genes that are upregulated in IFNβ and p19Arf+IFNβ treatments, cluster 2 represents genes that upregulated in p19Arf and p19Arf+IFNβ treatments, cluster 3 are genes downregulated in all treatments compared to GFP and cluster 4 represents genes that are upregulated in IFNβ and p19Arf+IFNβ treatments, but with less intensity.

transduced with pGreenFire1-ISRE were sorted (two rounds), based on GFP expression, resulting in the B16ISRE-GFP-Luc cell line.

Construction of adenoviral vectors

Our group has a set of adenoviral vectors constructed following Gateway technology (Invitrogen), as described previously.[20] In this system, we constructed desired expression cassettes in an entry vector (denominated pENTR) and, by site-directed recombination, transfer these cassettes to adenoviral vectors (denominated pAd/PL-DEST). We received from Dr Hiroyuki Mizuguchi (Osaka University, Japan) a modified adenoviral vector containing the RGD motif (between residues threonine-546 and proline-547 of the fiber protein) that follows Adeno-X technology (Clontech, Mountain View, CA, USA).[45] In order to render this vector compatible with the site-directed recombination strategy and our existing pENTR vectors, extensive modifications were made. First, we removed the SV40, polyA and CMV sequences from pShuttle2 (Clontech) and then we inserted, between *Not* I sites in this vector, the Gateway sequence that was amplified from

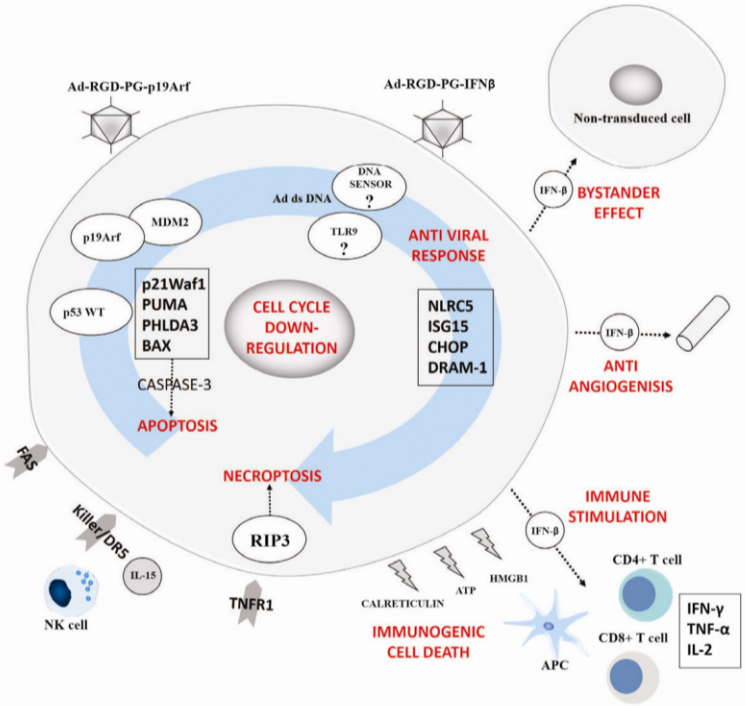

Figure 7. Schematic representation of the proposed mechanism of action of combined p19Arf and IFNβ gene therapy in B16 cells.

pAd/PL-DEST. Then, the Gateway sequence was removed from the new pShuttle vector and inserted in AdRGD vector, by *PI-Sce* I and *I-Ceu* I digestions. Once this was made, we could transfer, by site-directed recombination between pENTR (Invitrogen) and the new AdRGD-DEST vector, our expression cassettes containing the PG promoter, transgene of interest and a polyA sequence. The set of AdRGD-PG vectors constructed includes three monocistronic vectors, containing the eGFP or the p19Arf or the murine IFNβ genes, and one bicistronic vector, which contains both p19Arf and IFNβ genes separated by the IRES sequence described by Ghattas *et al.*[46] All vectors express the transgenes under control of a p53-responsive promoter, called PG, previously described by Bajgelman and Strauss.[23] Cloning details are available upon request.

Adenovirus production

Virus production was performed as described previously.[47,48] The biological titer was determined using the Adeno-X Rapid titration kit (Clontech). For transduction of target cells, the MOI was calculated based on the biological (infectious) titer.

In vitro assays

To maintain brevity, detailed procedures for several assays (X-Gal staining, immunofluorescence detection of p19Arf, enzyme-linked immunosorbent assay for detection of IFNβ, flow cytometric assessment of cell cycle, AnnexinV/PI staining, flow cytometric detection of eGFP, growth curve and cell viability assay) will not be presented here since they have been described previously,[8,20,21,24] though additional information is available upon request.

In situ treatment of B16 tumors using the adenoviral vectors

C57BL/6 mice (7 week old, female) were obtained from the Centro de Bioterismo, FMUSP and were maintained in SPF conditions, with food and water *ad libidum*. One million B16 cells (parental, non-modified) were inoculated in the left flank of C57BL/6 mice and, after establishment of the tumor (10 days), mice were treated with three intratumoral injections of 5×10^8 infectious units of AdRGD-PG vectors in a volume of 25 μl, every 2 days. Tumor size was determined by measurement with a digital caliper or mice were killed and tumors were collected 48 h after the last injection. Tumors were fixed for 24 h in 4% PFA, immersed in 30% sucrose and frozen before tissue sectioning with a cryostat and immunofluorescence detection of LC3β or RNA extraction. For RNA extraction, 100 mg of samples were lysed with a digestion buffer (200 mM TRis-HCl, 200 mM NaCl, 1.5 mM $MgCl_2$, 2% SDS and 500 μg/ml of proteinase K, pH 7.5) at 60 °C overnight and RNA were extracted by Trizol reagent (Invitrogen) following the fabricant's instructions. RT-qPCR reactions were performed as described later.

In other experiment, 1×10^6 B16ISRE-GFP-Luc cells were inoculated in the left flank of C57BL/6 mice and mice were treated with AdRGD-PG-eGFP or AdRGD-PG-IFNβ vectors, as described above. One day after the last injection, mice were killed and tumors were collected for dissociation with RPMI containing 35 μg/ml of liberase (Roche, Mannheim, Germany) and fixed with 4% PFA for cytometric analysis of GFP expression or lysed for analysis of luciferase expression, measured with Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions and using microplate reader Victor (Perkin-Elmer, Waltham, MA, USA).

All procedures and conditions were approved in accordance to the guidelines of animal care and use by the Scientific and Ethics Committee of the Cancer Institute of São Paulo, University of São Paulo School of Medicine.

Analysis of IFNβ bystander effect *in vitro*

In the co-culture experiment, B16 or B16ISRE-GFP-Luc cells were transduced as described for the growth curve and replated in six-well dishes with 1×10^5 cells total. For the mixtures, we combined in the same dish 1×10^4 cells transduced with one virus plus 9×10^4 cells transduced with a different virus, achieving a proportion of 1 : 9 of the mixture. For controls, we plated 1×10^5 cells of each transductions made previously. Cells were collected 72 h after the transductions for analysis of cell cycle or GFP expression by flow cytometry (FACScan, Becton Dickenson, San Jose, CA, USA).

Treatment with nutlin-3, IFN-R and Poly (I:C)

Cells were plated 1×10^5 cells/well in six-well dishes, transduced the next day with a MOI of 500 of the virus and/or treated with nutlin-3 (Sigma-Aldrich, St Louis, MO, USA), recombinant mouse IFNβ (R&D Systems, Minneapolis, MN, USA) and/or Poly (I:C; Sigma-Aldrich). For treatment with Poly (I:C), cells were transfected using lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Cells were collected 72 h after transduction for cytometric analysis of cell cycle alterations. In parallel, supernatant of cells transfected with Poly (I:C) was collected for detection of IFNβ by ELISA, as described elsewhere.[20]

Reverse transcriptase quantitative PCR

Cells were plated 1×10^5 cells/well in six-well dishes, transduced the next day with a MOI of 500 of the virus and collected 36 h later with 0.5 ml of Trizol reagent (Invitrogen) using a cell scraper. Total RNA was extracted following the fabricant's instructions and RNA concentration was determined by measuring absorbance at 260 nm. Extracted RNA quality was accessed by the protein and salt concentration, and by visualizing the 18 and 28S ribosomal RNA bands in a 1% agarose gel. Primers (Supplementary Table S1) were designed on the basis of the coding region, using the software Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers are complementary to sequences present in different exons (separated by introns), as predicted in sequences deposited at http://www.ncbi.nlm.nih.gov/nucleotide. Two different reference genes were tested (β-actin and glyceraldehyde 3-phosphate dehydrogenase), and β-actin was determined to be the most stable among the samples. Efficiency of all designed primers was validated, being at minimum close to 100%. Total RNA (2 μg) was reverse transcribed using random primers and moloney murine leukemia virus reverse transcripatse (Invitrogen). Reaction conditions were 100 ng cDNA (final volume of 10 μl); 12.5 μM of each primer; 5 ml of Syber Green PCR Master Mix (Invitrogen). Amplification conditions consisted of denaturation at 95 °C for 15 min, followed by 40 cycles denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. All samples were tested in duplicate and analyzed by the 7500 Fast Software, version 2.05 (Applied Biosytems, Foster City, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used for gene expression quantification, and data are presented as compared with the non-transduced B16 condition.

Western blot

Cells were plated 1×10^6 cells in 100 mm dishes, transduced the next day with a MOI of 500 of each virus and collected 36 or 60 h after transduction and subjected to western blotting, as described previously.[20] The blotted membranes were blocked with 5% non-fat milk for 1 h at room temperature and probed with the following antibodies: anti-PARP #9542 diluted at 1 : 1000 (Cell Signaling, Danvers, MA, USA), anti-Bax #sc-23959 diluted at 1 : 500 (6A7, Santa Cruz Biotechnology Europe, Heidelberg, Germany) and anti-RIP3 #sc-47364 diluted at 1 : 500 (C-16, Santa Cruz Biotechnology Europe). For the secondary antibody, a horseradish peroxidase-conjugated antibody was used and revealed using the ECL detection kit (GE Healthcare Life Sciences, Marlborough, MA, USA).

Flow cytometric analysis of CRT exposure

Cells were plated 1×10^5 cells/well in six-well dishes, transduced the next day with a MOI of 500 of the virus and collected after 72 h for immunostaining with a CRT-specific antibody (#NB300-545, Novus Biologicals, Littleton, CO, USA). After cells were washed with PBS and stained with Alexa488-conjugated anti-rabbit secondary antibody (Thermo Fisher, Rockford, IL, USA) for analysis by flow cytometry (FACScan, Becton Dickenson).

Measurement of released ATP by cells

Cells were plated 1×10^5 cells//well in six-well dishes, transduced the next day with a MOI of 500 of the virus and supernatant was collected 72 h after for ATP measurement. All cells (adherent and in supernatant) were collected in order to determine total protein concentration by Bradford assay with BSA standard. ATP concentration was measured by ENLITEN ATP Assay System (Promega) and normalized with protein concentration of the sample. Bioluminescence detection was made with microplate reader Victor (Perkin-Elmer).

Measurement of HMGB1 release

Cells were plated 1×10^5 cells/well in six-well dishes, transduced the next day with a MOI of 500 of the virus and supernatant was collected 72 h after for HMGB1 measurement as described by HMGB1 ELISA kit (IBL International GMBH, Hamburg, Germany). All cells (adherent and in supernatant) were collected in order to determine total protein concentration by Bradford assay with BSA standard and HMGB1 concentration was normalized with protein concentration of the sample.

Immunofluorescence detection of LC3β

Tumor sections were blocked with bovine serum albumin, probed with a polyclonal antibody for LC3β #sc-16755 diluted 1 : 500 (N-20, Santa Cruz Biotechnology Europe) followed by an anti-goat secondary antibody labeled with Alexa-594 diluted 1 : 3000 (Molecular Probes, Eugene, OR, USA). Nuclear staining was performed with Hoechst 33258, 20 μg/ml (Molecular Probes). Cells were visualized by confocal microscopy at × 100 amplification.

Microarray analysis

Cells, 8×10^5, were plated in 100 mm dishes and transduced 24 h later with a MOI of 500 of AdRGD-PG vectors in 4 ml of RPMI 10% FBS medium. Four hours later, 6 ml of RPMI 10% FBS medium were added and cells were maintained for additional 32 h. Then, adherent cells and cells in suspension were collected for RNA extraction. RNA extraction was performed using TRIzol reagent (Thermo Scientific, Waltham, MA, USA, 15596026) following the manufacturer's instructions. RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA, G2939AA). All the samples that had a RIN equal to or greater than eight were profiled on microarrays. Gene Chip Mouse 1.0 ST (Affymetrix Inc., 901171) was used according to the manufacturer's instructions to determine gene expression. Microarray results from three biological experiments were analyzed using TM4 Microarray software suite (Dana-Farber Cancer Institute, Boston, MA, USA). Differential gene expression profile was obtained by comparing the p19Arf, IFNβ and p19Arf+IFNβ to eGFP-treated cells using the significance analyses for microarray (false discovery rate $< 1\%$). K-means clusters were designed using Euclidian distance. Hierarchical cluster was designed using Euclidian distance and complete linkage. Bootstrap was used to evaluate dendrogram's consistency. Enrichment analysis was performed in DAVID database (EASE score < 0.05). Data can be accessed through Annotare repository (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5399).

Statistical analysis

The GraphPad Prism 5 software (La Jolla, CA, USA) was used for statistical analyses. All comparisons were conducted through one-way analysis of variance, followed by Kruskal–Wallis or Tukey's multiple comparison; two-way analysis of variance, followed by Bonferroni; or *t*-test. *P*-value lower than 0.05 was considered significant.

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AUTHOR CONTRIBUTIONS

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COMPETING INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

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7

Potentiation of p19Arf and interferon-beta immunotherapy through its association with doxorubicin: combining two immunogenic cell death inducers for the treatment of cancer

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Ongoing study

ABSTRACT

Association of immunotherapies with immunogenic chemotherapy can significantly improve cancer treatment, either by enhancing cell death or by providing superior immune stimulation. However, combinatorial strategies struggle with an increase of severe treatment-related toxicity. Previously, we have developed specialized adenoviral vectors for the delivery of $p19^{Arf}$ (tumor suppressor protein) and Interferon-β (IFNβ, pleiotropic immunomodulatory cytokine), a unique gene transfer combination capable to promote immunogenic cell death (ICD) and antitumor immunity. And here, we aim to investigate potential benefits that underlie the association of p19Arf/IFNβ immunotherapy with doxorubicin (Dox) chemotherapy, also an inducer of ICD. I*n vitro*, this association strongly augments induction of cell death, what allows the use of a reduced number of viral particles as well as Dox concentration. *In vivo*, employing an intratumoral treatment model of established tumors, we noticed that this p19Arf/IFNβ and Dox association strongly benefits our gene therapy treatment and, remarkably, also restores efficacy of a sub-therapeutic dose of Dox, what in contrast to its therapeutic dose, does not impair cardiac function. Additionally, this association can also potentiate immunogenicity of treated cells, providing superior antitumor protection in a therapeutic vaccine model. In sum, the associated use of p19Arf/IFNβ immunotherapy with Dox, a standard of care treatment modality, provides the means to extend immune therapeutic benefit of our gene-based approach, while circumventing treatment side effects.

INTRODUCTION

The practice of combining different treatment modalities, such as chemotherapy, surgery, radiotherapy and targeted therapy is well established in the clinic management of cancer patients (1). Yet, the remarkable success obtained with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed and cell death protein 1 (PD-1) checkpoint blockade immunotherapy is imposing a change in this scenario, as targeting of these pathways is achieving durable responses in myriad different malignancies when applied as monotherapies and, even more striking, in combination (2-5).

These results indicate that these checkpoints have a non-redundant role in regulating the amplitude of the immune response and that combinatorial strategies have the potential to improve even further clinical benefit. But, caution is needed in light of the increase of severe treatment-related toxicity (6), which may pose a limit on how many checkpoints can be targeted safely and argues in favor of combinations that are not based only on checkpoint modulators (6). Among the strategies one can employ for this end, evidence indicates that association of immunotherapies with immunogenic chemotherapy can strongly augment therapeutic efficacy (7).

Immunogenic chemotherapy has been shown to use different mechanisms to modulate the immune system present within the tumor microenvironment (TME) (8-12), especially by the induction of Immunogenic Cell Death (ICD), which is characterized by a series of temporally regulated events that mediate the release of danger associated patterns (DAMPS) from dying cancer cells in order to stimulate robust maturation of antigen presenting cells (APCs) and eventually an effective priming of $CD8^+$ T cells (13). Such events include secretion of ATP (14), exposure of calreticulin from the endoplasmic reticulum (15), as well as the passive release of high-mobility group box 1 (HMGB1) (16). And although these ICD markers were initially identified as a cellular response to Anthracyclines, including doxorubicin (Dox) (17), additional chemotherapeutic agents as well as other classes of inducers have been identified, such as radiotherapy (16) and hypericin-based photodynamic therapy (18). Importantly, data indicate that each of these agents is endowed with a specific ability to elicit these ICD markers and to act on immunity by other mechanisms, besides ICD (19), making the association of different ICD inducers an interesting field of investigation.

In accordance with this notion, we have developed a set of replication-deficient adenoviral vectors (serotype 5) that encode the $p19^{Arf}$ (tumor suppressor protein, p53 functional partner) and murine Interferon-Beta (IFNβ, pleiotropic immunomodulatory cytokine) cDNAs under the control of a p53 responsive promoter, named as PGTxB (or simply PG). And more recently, we showed that the adenoviral vector mediated delivery of the $p19^{Arf}$ and IFN-β (p19Arf/IFNβ) combination to tumor cells that harbour wild-type p53 unleashes a cell death program that display features of necroptosis, while mediating the release of *bona fide* ICD markers as well as immune protection against tumor progression located at primary and distant sites (20-22). Indeed, in a previous study, we explored a prophylactic vaccine model and observed that dying B16 mouse melanoma cells, treated *ex vivo* with the p19/IFNβ combination, function as an anti-cancer vaccine when injected during the cell death process, provoking a $CD4^+$ and $CD8^+$ T cell-dependent attack against fresh naive B16 cells, injected in the opposite flank (23). We also explored an *in situ* gene therapy model and obtained supporting evidence on the superior immune stimulatory capability of our combination over the single use of IFNβ. Since only in the group that had its primary tumors co-transduced with the $p19^{Arf}$ and IFNβ adenoviruses did we notice a significant reduction in the progression of a contralateral secondary tumor challenge, indicating, just as the vaccine model, an immune advantage for the use of this gene combination (24).

Even so, we propose that therapeutic efficacy of our vectors could be further improved by exploring their association with other therapies and, as our first attempt on this matter, here we have investigated the potential benefits of associating p19Arf/IFNβ immunotherapy with Dox immunogenic chemotherapy, an inducer of ICD (17) frequently used for the treatment of sarcoma, lung, ovarian and other cancers (25).

Dox acts by disrupting the topoisomerase II-mediated DNA repair and by producing free radicals that inflict damage to several cellular components (25). Additionally, as previously reported by our group and others, treatment with Dox can lead to the activation of the apoptotic p53 transcriptional pathway (26). Even though its clinical use is considered as an effective anticancer agent, it is limited, in part, by the induction of severe cardiotoxicity, which depending on the dose, can evolve to chronic cardiomyopathy with high mortality rate (27, 28).

Therefore, taking the aforementioned evidence into consideration, we hypothesize that the association of Dox with our adenoviral based therapy could impact the p19Arf/IFNβ therapeutic function on two main levels: (i) potentiation of cell death

and (ii) on its immunomodulatory properties. This association could be advantageous for Dox as well, by allowing the use of lower doses that reduce related toxicity.

To test these potential benefits, we have considered two key aspects, the cell line and treatment order. Serving as a control for the cellular and molecular ICD mechanisms unleashed by Dox treatment, here we used the MCA205 (MCA) sarcoma cell line and employed an intratumoral (i.t) application model, since it was already demonstrated that under these conditions MCA cells succumb to ICD (17). We also used the B16F10 (B16) mouse melanoma cell line, as it was with this model that we revealed the cell death and immune stimulatory events of our p19Arf/IFNβ treatment. With regard to the treatment order, we based our approach on the work of Fridlender and collaborators (2010) that showed that association of an adenoviral vector encoding IFNβ with chemotherapy is more effective when immunotherapy is applied first (29).

Data presented here indicates that *in vitro* the association of p19Arf/IFNβ with Dox (p19Arf/IFNβ + Dox) drastically enhances cell death, allowing the application of a reduced number of viral particles as well as Dox concentration. *In vivo*, upon i.t treatment of established tumors, this association improves tumor control by our gene therapy, matching the efficacy of a therapeutic dose of Dox. But, on the other hand, pre-treatment with p19Arf/IFNβ enhances the effect of a sub-therapeutic dose of Dox and preserves cardiac function. Moreover, there is also indication of an increase in the immune stimulatory capability, by modulating secretion of *bona fide* ICD markers and providing superior antitumor protection in a therapeutic vaccine model. Therefore, use of p19Arf/IFNβ gene therapy in association with immunogenic chemotherapy fits the current criteria of combinatorial approaches and warrants further development.

METHODS

Cell culture and cell lines

The mouse cell lines MCA205 H-2b (MCA, methylcholanthrene derived sarcoma (kindly provided by Dr. João Paulo Catani, Ghent University, Belgium) and B16F10 (B16, melanoma, kindly provided by Dr. Roger Chammas, ICESP, Brazil) were maintained in a humidified incubator at 37 °C with 5% CO_2 and cultivated in Roswell Park Memorial Institute (RPMI) medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen) as well as 1x Anti-Anti (Antibiotic-Antimycotic, Gibco by Life Technologies). HEK293 cells (293A, Invitrogen, CA, USA) were cultivated in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented and maintained in the same conditions as above. The MCA-DEVD cell line was

generated by transduction with a lentivirus reporter for caspase-3 activity and selection for puromycin resistance (0.5μg/mL). This vector, described in (30), encodes a constitutively expressed luciferase-GFP protein separated from a polyubiquitin domain via a caspase-3 cleavage site and was generously provided by Dr. Chuan-Yuan Li (Department of Radiation Oncology, University of Colorado School of Medicine, Aurora, CO, USA).

Virus construction, production and titration

Construction and production of AdRGD-PG adenoviral vectors (serotype 5) containing modification with the RGD motif in the fiber as well as the p53-responsive promoter (PGTxβ, PG) was previously described in (20). Detection of $p19^{Arf}$ and murine IFN-β cDNAs was performed as described in (22). Titration of adenoviral stocks was performed using the Adeno-X Rapid Titer kit (Clontech) and titer yields were: AdRGD-CMV-LacZ ($3.6x10^9$ IU/mL, infectious units/milliliter), AdRGD-PG-LUC ($1x10^{11}$ IU/mL), AdRGD-PG-eGFP ($5x10^{10}$ IU/mL), AdRGD-PG-p19 ($1.3x10^{10}$ IU/mL) and Ad-RGD-IFNβ ($5x10^{10}$ IU/mL).

***In vitro* assays**

MCA or B16 cells ($1x10^5$) were plated in 6 well plates containing 1 mL of RPMI media and transduced with the desired adenovirus MOI (multiplicity of infection). After overnight transduction (12 to 16 hours), 2 mL of media was added and cells kept in culture until needed. When combing adenoviral transduction with chemotherapy, Dox (doxorubicin hydrochloride, Sigma) was added immediately after the overnight transduction using the concentration indicated for each experiment. Importantly, in the Dox single treatment condition, Dox was added at the same moment as in the association group, 12 to 16 hours after cell plating. After 12 hours treatment with Dox (1 mg/mL) or Nutlin-3 (10 μM, Sigma), expression of eGFP from AdRGD-PG-eGFP was analyzed by flow cytometry (Attune, Life Technologies). Cell viability was assessed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT assay where, 8 hours after transduction in 6 well plates, $2x10^4$ cells/well were plated in 96 well plates, treated with Dox, and analyzed after 16 hours of incubation. Non-transduced cells were used as viable control and protocol was carried out according to (31). Cell cycle analysis by propidium iodide (PI) staining was carried out 72 hours after p19Arf/IFNβ and Dox single treatment, as previously described in (22). Analysis of caspase 3 activity *in vitro* was made using the CellEvent caspase-3/7 green reagent (Molecular Probes by Life Technologies, USA) by flow cytometry, following manufacturer's instructions and 16 hours after combined treatment. Last, analysis of

ICD markers upon p19Arf/IFN-β + Dox was conducted as explained in detail in (20). Briefly, detection of calreticulin^{+} and PI^{-} cells was made 14 hours after combined treatment, by staining with a CRT-specific antibody (1:100, Novus, Biologicals) and after cells were washed with PBS, with Alexa488-conjugated anti-rabbit secondary antibody (1:500, Thermo Fisher) followed by PI staining to exclude dead cells, immediately before flow cytometry.

Central Composite Rotational Design

Central Composite Rotational Design (CCRD) was performed by employing two independent variables (i.e., p19Arf/IFNβ and Dox), rationally combining five different doses of each one (detailed in **Table 1**).

Table 1. Variables, levels and doses applied to Central Composite Rotational Design (CCRD).

Variables	Level/dose (codified point)				
	axial (-1.4)	**inferior** (-1)	**central** (0)	**superior** (+1)	**axial** (+1.4)
p19Arf/IFNβ (MOI)	196.5	300	550	800	903.5
Dox (μM)	5.5	8	14	20	22.5

According to **Table 2**, a total of twenty experimental conditions were designed, which encompass eleven main combinatorial assays (condition 01 to 12), plus five repetitions of the central point level in order to calculate the standard error (condition 13 to 17). Additionally, three controls were included: cells without treatment (condition 18) and treated with just p19Arf/IFNβ and Dox, each one at central level (conditions 19 and 20, respectively). The percentage of hypodiploid cells, accessed by PI staining after 20 hours of combined treatment, was considered as a readout to quantify cell death and analyzed by response-surface methodology (RSM). Importantly, the statistical comparison of variables was carried out by multivariate ANOVA, and a second-order polynomial regression was applied to represent the interaction between variables. A p-value less than 0.05 was considered to be statistically significant. These analysis were performed using the software *Statistica* v.12 (*Statsoft,* Inc., Tulsa, OK, EUA).

Table2. Experimental conditions investigated in the Central Composite Rotational Design (CCRD).

Condition		Codificated variables*		Experiemental points	
		p19/IFNβ (MOI)	Dox (µM)	p19/IFNβ (MOI)	Dox (µM)
Main factorial points	01	-1	-1	300	8
	02	+1	-1	800	8
	03	-1	+1	300	20
	04	+1	+1	800	20
	05	-1	Central point	300	14
	06	+1	Central point	800	14
	07	Central	-1	550	8
	08	Central	+1	550	20
Axial points	09	Central	-1.4	550	5.5
	10	Central	+1.4	550	22.5
	11	-1.4	Central	196.5	14
	12	+1.4	Central	903.5	14
Central points (replicates)	13	Central	Central	550	14
	14	Central	Central	550	14
	15	Central	Central	550	14
	16	Central	Central	550	14
	17	Central	Central	550	14
Controls	18	Untreated	Untreated	0	0
	19	Central	Untreated	550	0
	20	Untreated	Central	0	14

*more details in **Table 1**

***In vivo* gene therapy and doxorubicin treatment models**

MCA ($2x10^5$) or B16 ($5x10^5$) cells were harvested, washed twice with cold PBS, resuspended in 100 µL of PBS per mouse and afterwards, inoculated subcutaneously (s.c) in the left flank of immune competent C57BL/6 or immune deficient Balb/c Nude (*Foxn1n*) mice. On day 8, palpable tumors were treated three times, once every 2 days, with intratumoral (i.t) injections of the following adenoviral vectors, Ad-RGD-CMV-LacZ or Ad-RGD-PG-LUC ($4x10^8$ IU, resuspended in 25 µL final volume of PBS/mouse) or co-transduced with AdRGD-PG-p19 e AdRGD-PG-IFNβ ($2x10^8$ IU, for each vector and maintaining the 25 µL final volume per mouse). For the Dox single treatment model, chemotherapy was applied (i.t) a single time at day 12 with the following doses: 5, 10, 20, and 60 mg/kg (in the final volume of 30 µL of PBS/mouse). Whereas in the association model, adenoviral vectors were injected as explained above and Dox gave 2 days after the last viral injection (day 14), following

the injection method as the Dox single treatment group. Tumor progression was followed every two days and volume calculated as described in (23). For the survival analysis comparing C57BL/6 and Nude mice, treated mice were euthanized when tumor volume reached 1000 mm^3.

Both C57BL/6 and Nude mice were female, 7 weeks old, obtained from the *Centro de Bioterismo da Faculdade de Medicina da Universidade de São Paulo* (FMUSP) and kept in the animal facility in the *Centro de Medicina Nuclear* (CMN) in SPF conditions, with food and water *ad libitum*. The well-being of the mice was constantly monitored following the ethics committee guidelines from the FMUSP, protocol number 165/14.

Leucogram

Peripheral blood was collected from the mice at 3 and 5 days post-therapy by retro-orbital puncture and placed in tubes with EDTA before counting white blood cells at the *Laboratório de Medicina Laboratorial*, LIM 03, FMUSP.

Winn assay

Spleens from mice that had their MCA tumors treated *in situ* with GFP, p19Arf/IFNβ and/or Dox (20 mg/kg) were collected individually (10 days after the last treatment injection), passed through a 70μm cell strainer (Falcon, NC, USA), washed twice with PBS and red blood cells were lysed with ACK buffer (1.5 M NH_4Cl, 100 mM $KHCO_3$, and 100 mM EDTA-2NA) for 5 min. Then, viable splenocytes were counted with trypan blue exclusion, mixed with fresh untreated MCA cells ($1x10^6$ splenocytes: $1x10^5$ MCA cells) and inoculated in s.c in naïve C57BL/6 mice. Tumor volume was monitored and plotted on day 12 after adoptive transfer of cells.

***In vivo* bioluminescence imaging**

For the analysis of caspase 3 *in vivo*, MCA-DEVD tumors were treated *in situ* as described above and 24 and 48 hours after the last treatment injection, mice were submitted to bioluminescence imagining (IVIS Spectrum, Caliper Life Science) to detect the luciferase activity from the DEVD reporter. To this end, mice were injected via intraperitoneal (i.p) with 10 mg/kg luciferin (Promega) anesthetized with isoflurane (Cristalia) using the Xenogen anesthesia system before imaging. Images were captured and only the strongest signal from each tumor was included in the analysis with Living Imaging 4.3 software (Caliper Life Science). Luciferase activity was obtained from the average radiance value[p/s/cm^2/sr]. To calculate the fold activity overtime, average radiance values obtained for each mouse 48 hours post-treatment were divided by its

respective value at 24 hours. Parental MCA tumors were used as negative control and no emission was detected (data not shown).

For the analysis of AdRGD-PG-LUC expression, MCA tumors were injected three times, once every two days, with $2x10^8$ IU and 72 hours after the last injection, mice were submitted to IVIS Spectrum analysis of luciferase activity, just as explained above. An untreated MCA tumor was used as negative control and no emission was detected (data not shown).

Echocardiographic assessments

The systolic cardiac function was assessed by echocardiography. Exams were performed 10 days after treatments with AdRGD-PG-eGFP (adenovirus control), Dox 10 mg/kg, Dox 20 mg/kg and p19Arf/IFNβ + Dox 10 mg/kg. Mice were anesthetized with 1.5 to 2.5% isoflurane (in 100% oxygen ventilation). They were trichotomized and placed in supine decubitus to obtain cardiac images. Parasternal-long and short axis images were captured using VEVO 2100 ultrasound equipment (VisualSonics Vevo 2100 Imaging System, Canada) with a 40 MHz linear-transducer. Analyzes were performed off-line using VisualSonics software (VevoCQ LV Analysis, VisualSonics, Canada). Parameters such as Systolic and Diastolic volumes were calculated using Simpson's modified algorithms present in the Analysis software (parasternal-long axis images). Based on these volumes, stroke volume (μL) and left ventricle ejection fraction (LVEF, %) were calculated. Also, linear measurements were obtained from parasternal-short axis images. Left ventricle shortening fraction (LVSF, %) was calculated, using systolic and diastolic diameters. Still, left ventricle mass (LV mass, mg) was estimated by linear measurements. Beating rate (beats per minute – BPM) was calculated directly by an animal table-ECG system connected to VEVO 2100 system. Echocardiographic results were interpreted considering the American Society of Echocardiography recommendations concerning the mice model (32). All parameters were shown as the mean values of three consecutive cardiac cycles. Transthoracic echocardiography image acquisition and analysis was performed by an expert investigator who was blind to the experimental groups.

Vaccination model

In the first step of the therapeutic vaccine model, naïve C57BL/6 mice were inoculated (s.c) in the right flank (tumor challenge site) with fresh untreated MCA ($2x10^5$) or B16 ($6x10^4$) cells and in the second step, vaccinated (s.c) on days +3, +9 and +15 with $3x10^5$ *ex vivo* treated cells applied in the left flank (vaccine site). *Ex vivo* treatment was carried out as follows, MCA or B16 cells were seeded in 10 cm

plates with 2 mL of media and co-transduced with the AdRGD-PG-p19 and AdRGD-PG-IFNβ (MOI 500 for each) for 4 hours before the addition of 8 mL of fresh media. Then, cells were kept in culture for 16 hours and in the p19Arf/IFNβ + Dox or Dox groups, Dox (14 µM) was added for 6 hours, until cells were harvested, washed twice with cold PBS, counted and resuspended in 100 µL of cold PBS. For the DEAD cell + GFP control group, cells were transduced with the AdRGD-PG-eGFP vector (MOI 1000) and after 16 hours, harvested, washed twice with cold PBS, resuspended and lysed by three cycles of freezing and thawing. Whereas for the prophylactic vaccine model, mice were first vaccinated two times, once every seven days, with *ex vivo* treated B16 cells ($3x10^5$ cells) and 7 days after the last vaccine, challenged with fresh untreated B16 cells ($1x10^5$) injected in the opposite side. Progression at the vaccine and challenge sites was monitored as described above.

Checkpoint blockade immunotherapy

During the therapeutic vaccine model, C57BL/6 mice were inoculated (i.p) with 200 µg of the anti-CTLA-4 antibody (BioXcell, clone 9D9, IgG2b) on day 3 and with 100 µg on days 6 and 9, after inoculation of fresh B16 cells ($8x10^4$ or $6x10^4$ cells, day 0) in the challenge site. Alternatively, mice were also treated with 200 mg of anti-PD-1 antibody (rat chimeric murine IgG1 antibody, clone 4H2, Leinco Technologies) on days +3, +6 and +9 post-tumor transplant.

Statistical analysis

Data were presented as mean ± SEM. Statistical differences between groups was indicated with *p* values, being $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Statistical tests are indicated in each figure legend along with the number of independent experiments performed or number (n) of mice in each group. These analyses were made using the GraphPad Prism 5 (La Jolla, CA, USA) software, with exception of the CCRD analysis (explained above).

RESULTS

Use of p19Arf/IFNβ and doxorubicin as monotherapies

The set of adenoviral vectors used in this work, called AdRGD-PG, feature the RGD modification in the fiber, which allows efficient transduction of target cells by using heparin sulfate and integrin α_v, without depending on the coxsackievirus and adenovirus receptor (CAR) (33). Additionally, transgene expression is under the control of the PG promoter, which under the control of p53 achieves expression levels superior to those seen with traditional constitutive promoters and also functions as a monitor of

p53 transcriptional activity (26, 34, 35). Considering that the response of the MCA cell line to our AdRGD-PG-p19 and AdRGD-PG-IFNβ vectors was not investigated in previous studies by our group, we first aimed to characterize if this cell line could be efficiently transduced and drive expression of the PG promoter in response to well-known stimuli of p53 activity. To this end, MCA cells were transduced with increasing MOI (100 to 1000) of the AdRDG-PG-eGFP vector and also treated with Dox and Nutlin-3. As shown in **Figures 1 a and 1 b,** MCA cells are effectively transduced and can increase GFP fluorescence by two-fold upon treatment with either Dox or Nultin-3, thus suggesting that this cell line is endowed with transcriptionally functional p53 capable of driving expression from the PG promoter. Indeed, co-transduction with the AdRGD-PG-p19 and AdRGD-PG-IFN-β vectors (p19Arf/IFNβ) augments cell death levels in comparison with their individual treatments or the Lac-Z control vector **(Figure 1 c)**, supporting previous observations made with the B16 and LLC-1 cell lines (22, 24).

Employing an *in situ* gene therapy model, where established MCA tumors are treated with intratumoral injections of adenoviral vectors, we show through the delivery of the AdRDG-PG-LUC vector that these tumor cells are also transduced *in vivo*, as indicated by the luciferase signal detected within the tumor mass **(Figure 1 d)**. Next, the antitumor effect of p19Arf/IFNβ treatment was compared between C57BL/6 immune competent and Nude immune-deficient mice, revealing that significant reduction in tumor progression **(Figure 1 e)** as well as increased survival **(Figure 1 f)** require involvement of the adaptive immune response, at least to some extent.

We also analyzed the effect of Dox immunogenic chemotherapy as a monotherapy. *In vitro*, MCA cells were significantly killed by Dox in a dose-response manner **(Figure 1 g)**. Whereas *in vivo*, upon a single intratumoral injection of different doses of Dox, it was noticed that both the dose of 20 mg/kg and 60 mg/kg can inhibit tumor progression **(Figure 1 h)** and prolong survival **(Figure 1 i)**, especially when therapy is performed in an immune competent host. Since the dose of 5 and 10 mg/kg were not effective in inhibiting tumor progression, they will be considered hereafter as a sub-therapeutic doses. In accordance with the literature, therapeutic efficacy was hampered in immune-deficient mice, especially with the dose of 20 mg/kg, evidencing the importance of engaging the immune system for achieving therapeutic benefit (17, 36). Taken together, these results reveal the efficacy of p19Arf/IFNβ and Dox when applied as monotherapies and prompted us to investigate their association.

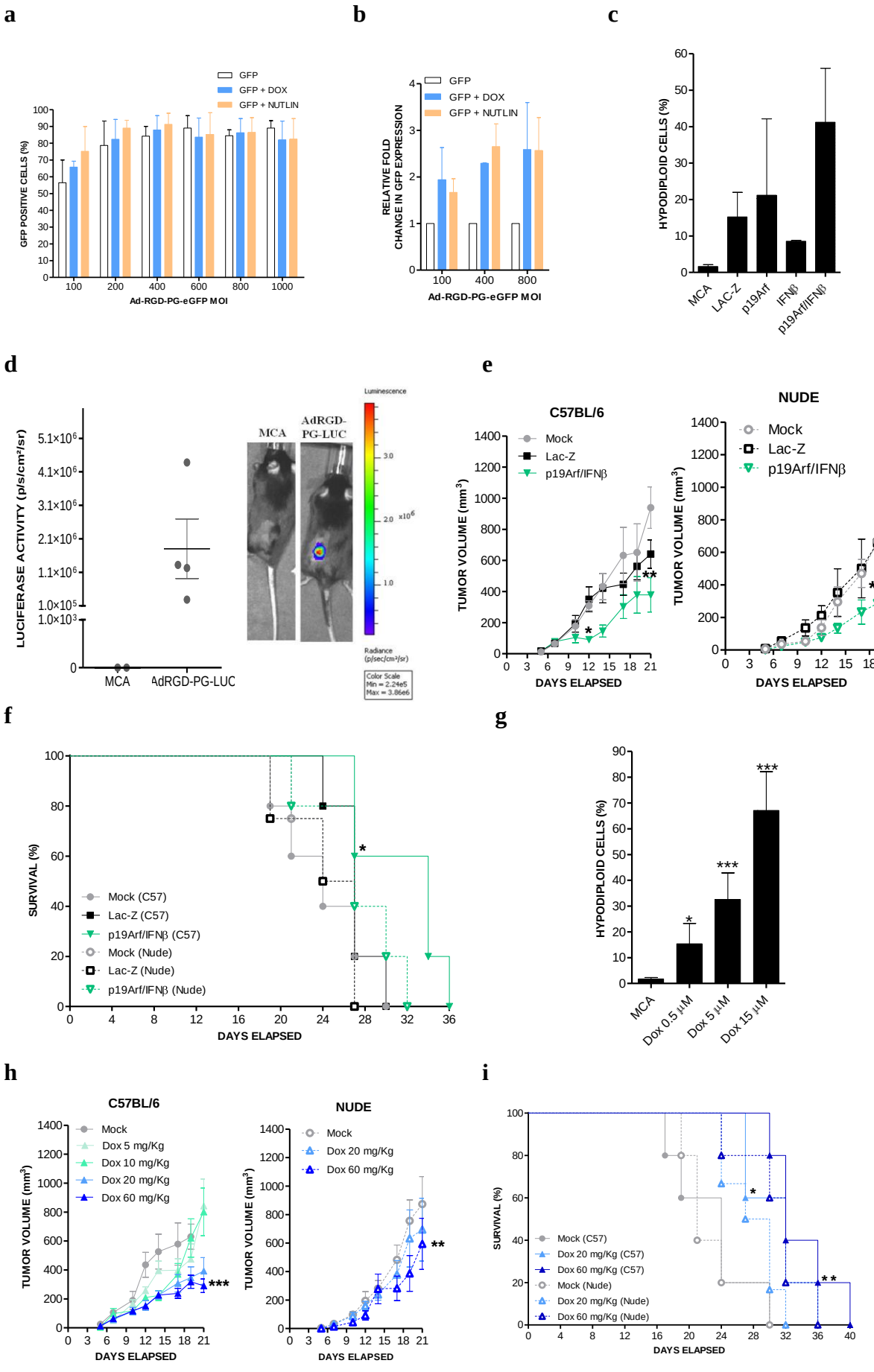


Figure 1. Use of p19Arf/IFNβ and doxorubicin as monotherapies inhibits progression of established MCA tumors in immune competent hosts. (a) Flow cytometry analysis of GFP positive MCA cells, as

well as, GFP fluorescence levels **(b)** upon AdRGD-PG-eGFP vector transduction and after 12 hours of doxorubicin (1 mg/mL) and Nutlin-3 (10 μM) treatment. n=*2*. **(c)** Induction of cell death 72 hours after *in vitro* transduction of MCA cells with AdRGD-PG-LacZ (MOI 1000), AdRGDPGp19 (MOI 500), AdRGDPGIFNβ (MOI 500) or co-transduction with AdRGDPGp19 and AdRGDPGIFNβ vectors (MOI 500 for each. n=2. **(d)** Bioluminescence imagining of MCA tumors 72 hours after intratumoral transduction with AdRGD-PG-LUC vectors. n=4. **(e)** Progression of MCA tumors in C57BL/6 and Nude mice upon *in situ* p19Arf/IFNβ gene therapy. [Two-way ANOVA and Bonferroni post-test]. **(f)** Survival analysis comparing immune competent C57BL/6 mice and immune deficient Nude mice [Log Rank Mantel-cox test, followed by Wilcoxon post- Test]. n=5 for the p19Arf/IFNβ (C57), Lac-z (C57), Mock (C57, p19Arf/IFNβ (Nude), n=4 for Mock (Nude) and Lac-Z (Nude) groups. **(g)** Induction of cell death 72 hours after treatment of MCA cells with doxorubicin. n=3 [One-way ANOVA and Tukey's multiple comparison post-test] **(h)** Progression of MCA tumors treated intratumorally with PBS (Mock) or doxorubicin and performed in C5BL/6 and Nude mice. [Two-way ANOVA and Bonferroni post-test]. **(i)** Survival analysis comparing immune competent C57BL/6 and immune deficient Nude mice. [Log Rank Mantel-cox test followed by Wilcoxon post- Test]. n=6 for the Mock (C57), Mock (Nude), Dox 60 mg/kg, Dox 10 mg/kg and Dox 5 mg/kg (C57) groups; n=5 for Dox 20 mg/kg (C57), Dox 20 mg/kg (Nude) and Dox 60 mg/kg (Nude).

In vitro association of p19Arf/IFNβ and doxorubicin

One key feature of the association between p19Arf/IFNβ and Dox would be its capability to synergistically increase the induction of cell death, while allowing a reduction in both virus MOI and Dox concentration. To test this hypothesis, cell viability of MCA and B16 cells was analyzed after exposure to increasing doses of Dox (1 to 30 μM) as well MOI (100 to 1100), applied in association or individually. Remarkably, a dramatic reduction in cell viability was observed in either cell line where combined treatment surpassed the effect caused by monotherapies, as noted by the lower doses of the combination (MOI 300 with 6 μM of Dox) in contrast to the higher doses employed for the single agents (MOI 1100 or 30 μM of Dox). In support of this evidence, we next performed a combinatorial experiment based on a central composite rotational design to evaluate the impact of Dox concentration and viral MOI on promoting cell death**.** MCA and B16 were treated with twenty different combinations of chemotherapy and p19Arf/IFNβ **(detailed in Table 2)** and the percentage of hypodiploid cells projected in a surface response graph **(Figure 2 b).** Data analysis from ANOVA indicates that both variables are statistically significant to response ($p<0.05$, data no-show), as well as the assumed regression models for both cell lines, which were valid for represent the response pattern of cell death. Through the analysis of surface response graphs, in which the interactions between the variables could be visualized by changes in the spectrum color as well the curvature of the lines, the p19Arf/IFNβ and Dox association was shown to enrich the induction of cell death. Interestingly, this enrichment was noticed even in conditions where Dox concentration and virus MOI are decreased in an interchangeable manner, indicating that by sensitizing cells to Dox chemotherapy, treatment with p19Arf/IFNβ allows use of lower doses with potentially

less severe side effects. Furthermore, aiming to obtain mechanistic insight on the molecular mechanism of cell death, we investigated the activity of caspase 3, known for playing a central role in the execution-phase of apoptosis (37) and on base of our previous study, is not involved in the necroptotic cell death induced by p19Arf/IFNβ (20). Accordingly, in both cell lines, treatment with just p19Arf/IFNβ provided little caspase 3/7 activity, in sharp contrast to 14 µM Dox, that displayed more than 40% of positive cells, which, strikingly, was further increased to 60% in the MCA cell line upon p19Arf/IFNβ+Dox treatment, suggesting an additive effect that may explain why this association strongly induces cell death, especially interesting in the case that the necroptotic machinery is still activated, a question we are currently investigating **(Figure 2 c)**.

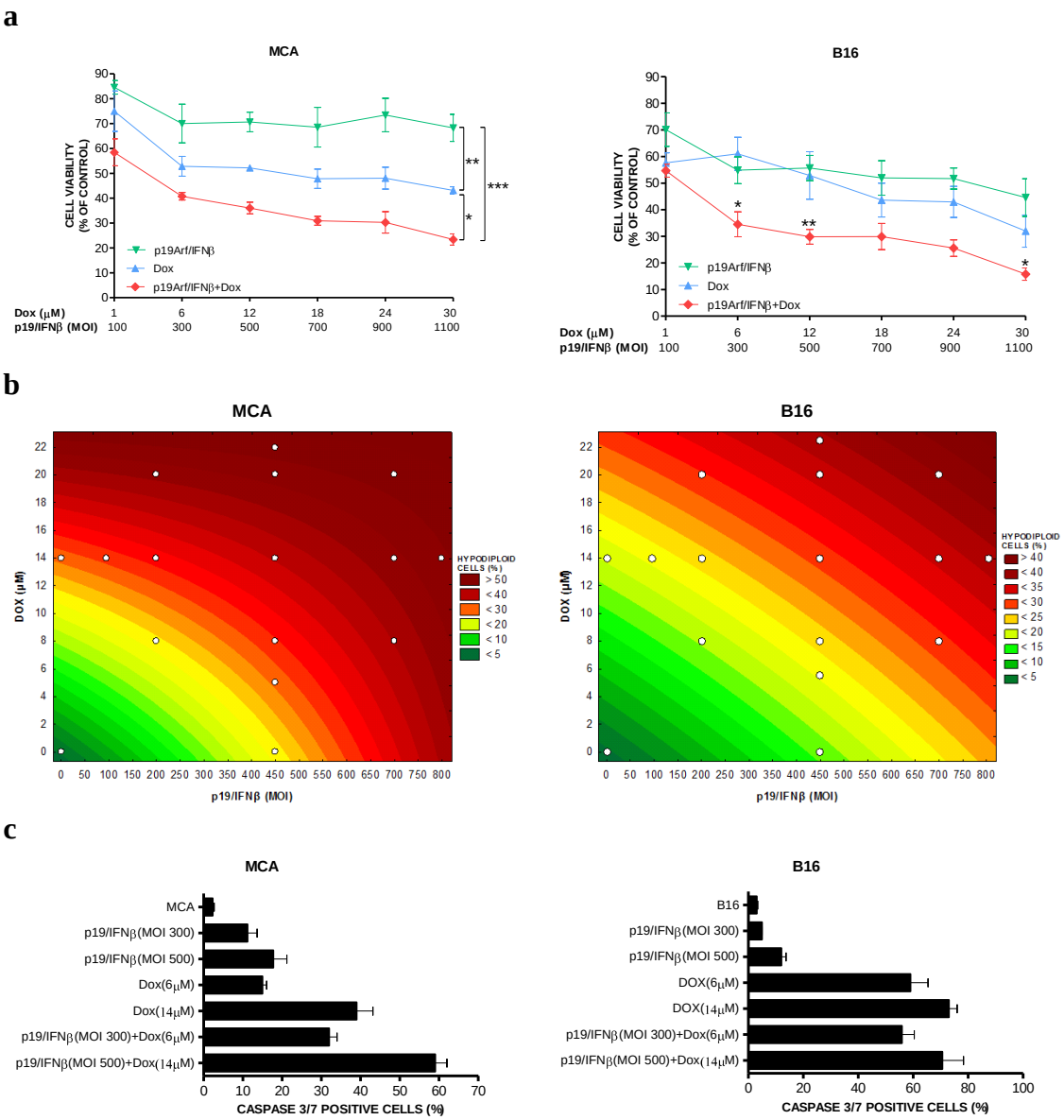


Figure 2. *In vitro* association of p19Arf/IFNβ and doxorubicin augments cell death levels. (a) MTT analysis of MCA and B16 cells treated for 12 hours individually or in combination with doxorubicin and AdRGD-PG-p19 and AdRGD-PG-IFNβ vectors. n=4 [One-way ANOVA and Tukey's multiple comparison post-test]. **(b)** Combinatorial experiment (central composite rotational design analysis) in

which MCA and B16 were treated for 20 hours with different MOI of p19Arf/IFNβ and concentrations of doxorubicin, data is represented by the percentage of hypodiploid cells in surface response graphs, the white dots represent each combinatory experimental point, the spectrum color indicate changes in the response (i.e. cell death), and the curvature of the lines show the response pattern as well as the interaction between variables. The central point (i.e. 14 μM Dox and 450 MOI p19Arf/IFNβ) were performed in quintuplicate; the data and regression model were analysed by multivariate ANOVA **(c)** Flow cytometry analysis of caspase 3 activity in MCA and B16 cells after 16 hours of p19Arf/IFNβ and of doxorubicin treatment. n=2.

In vivo association of p19Arf/IFNβ and doxorubicin

Next, we sought to investigate *in vivo* the therapeutic impact of associating p19Arf/IFNβ with Dox in comparison with the monotherapies. To this end, established MCA-DEVD tumors, which were stably modified to expresses a caspase 3 reporter, were pre-treated with three rounds of AdRGD-PG-p19 and AdRGD-PG-IFNβ vectors and two days later, also injected with the therapeutic dose of Dox, 20 mg/kg. From this assay, we noticed that individual treatments with p19Arf/IFNβ and Dox similarly reduce tumor progression when compared to the GFP control treatment **(Figure 3 a)**. However, as the tumor progresses, this similarity is lost, since tumors treated with just p19Arf/ IFNβ begin to grow more than Dox-treated tumors. The combined use of these treatments was strikingly effective not only in decreasing tumor volume but also in conferring a survival benefit **(Figure 3 b)**. Interestingly, only in the association group, complete regression was observed in one mouse, evidenced by reduced volume shortly after treatment, followed by a 40 day period of stable volume, then to complete regression on day 70, a result not demonstrated in the graph of tumor volume, but contemplated in the survival curve. In this way, treatment with Dox and its association with p19Arf/IFNβ were the most effective modalities for increasing survival **(Figure 3 b)**. Moreover, through the analysis of caspase 3 activity *in situ*, there was no difference in luminescence activity between these groups **(Figure 3 c)**. Yet, interestingly, when comparing the luciferase activity captured 24 and 48 hours after treatment **(Figure 3 d and e)**, we noticed that the GFP group presented a relatively constant luminescence intensity (fold of -0,157) and the p19Arf/IFNβ group had a -1.08 fold reduction in signal intensity after 48 hours. On the other hand, tumors treated with just Dox or its association with p19Arf/IFNβ, showed 2.2 and 1.13 fold increases, respectively. This assay suggests that cell death occurs in a dynamic manner, where pretreatment with p19Arf/IFNβ, just as *in vitro*, does not activate caspase 3, but upon subsequent treatment with Dox, apoptosis is strongly induced and an increase in luciferase activity is now detected. However, additional experiments are needed to confirm if levels of cell death are actually higher *in vivo* with the association.

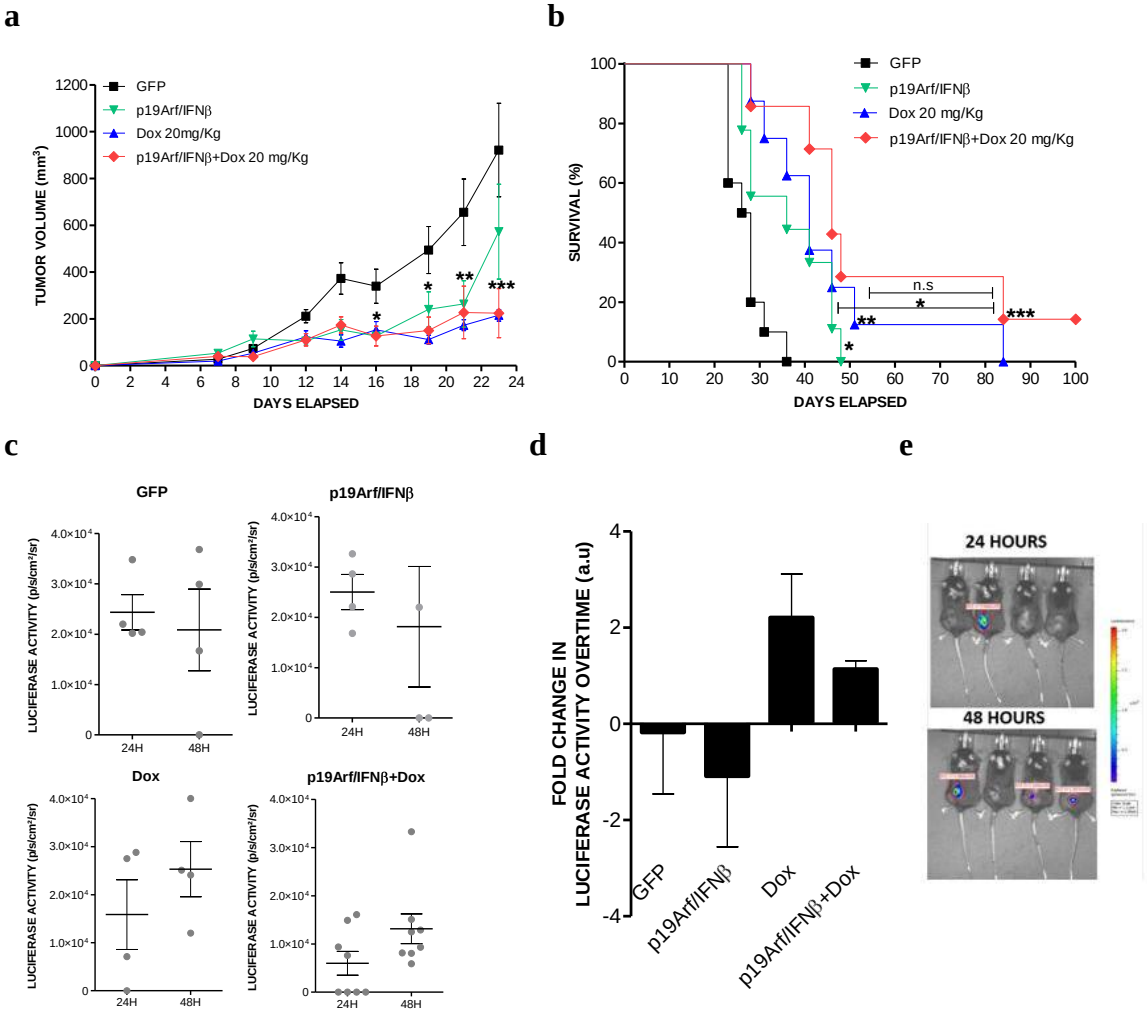
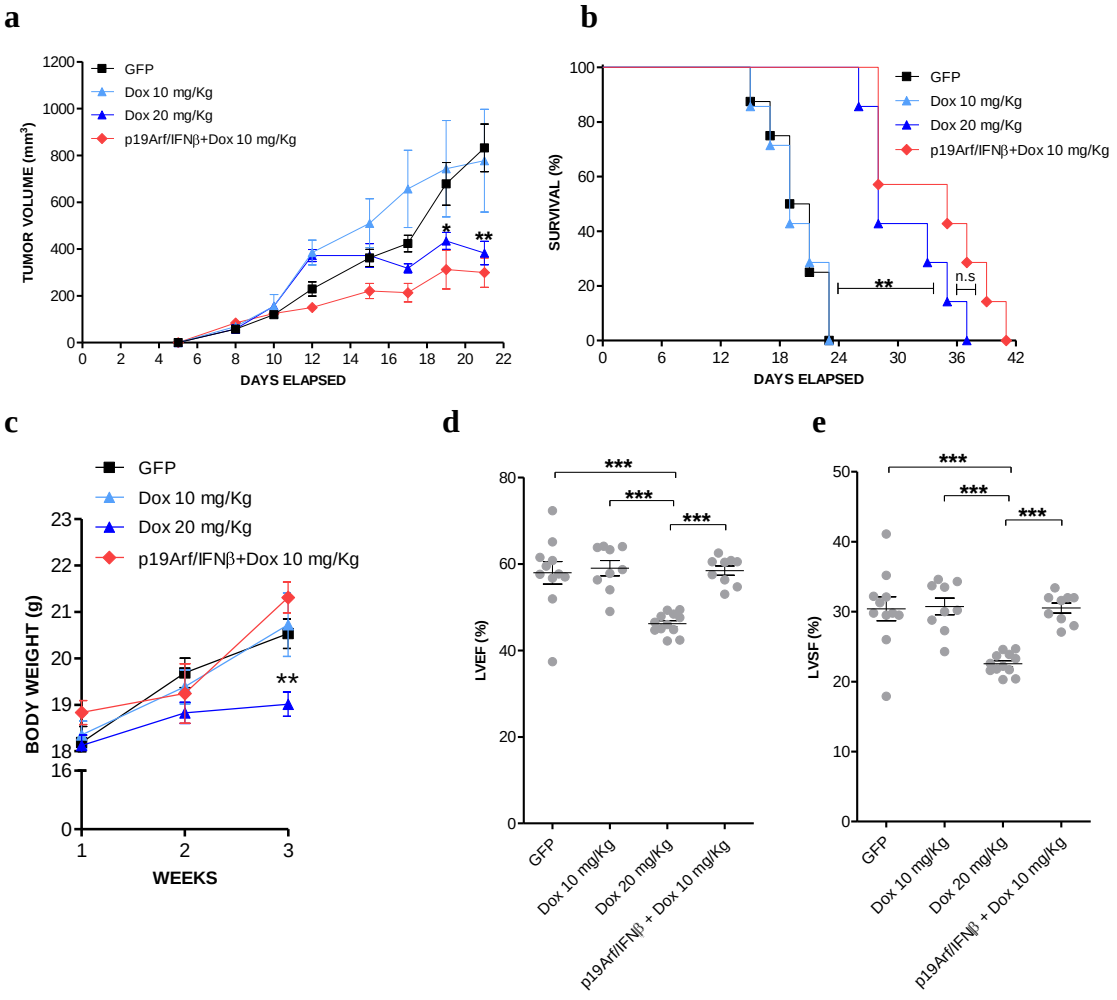


Figure 3. ***In vivo*** **association of p19Arf/IFNβ gene therapy with doxorubicin inhibits tumor progression and changes the dynamic of caspase 3 activity**. **(a)** Tumor progression curves of established MCA-DEVD tumors that were co-transduced with the AdRGD-PG-p19 and AdRGD-PG-IFNβ vectors and after 48 hours, also treated with 20 mg/kg of doxorubicin. [Two-way ANOVA and Bonferroni post-test] n=6 for all groups. **(b)** Survival analysis of mice from **a**. [Log Rank Mantel-cox test, followed by Wilcoxon post- Test. n=8 for all groups, except p19Arf/IFNβ which n=9 **(c)** Bioluminescence imaging of caspase 3 activity *in vivo* on MCA DEVD tumors treated as mentioned in **a**. n=4 for the GFP, p19Arf/IFNβ, and Dox groups. N=8 for the p19Arf/IFNβ+Dox group. **(d)** Fold difference in luciferase activity measured in **c**, between 48 and 24 hours after treatment. **(f)** Representative bioluminescence image of the p19Arf/IFNβ+Dox group.

Having observed that the association between p19Arf/IFNβ gene transfer with the therapeutic dose of Dox does not further reduce tumor volume as compared to Dox alone, we next asked whether the benefit of this association would maintain when using a sub-therapeutic dose of Dox, 10 mg/kg, and consequently ease induction of cardiotoxicity, a major side effect observed in the clinical setting. Therefore, after *in situ* pre-treatment with AdRGD-PG-p19 and AdRGD-PG-IFNβ adenoviral vectors, MCA tumors were injected (i.t) with 10 mg/kg of Dox, which when used individually does not reduce tumor progression to the same extent as the dose of 20 mg/kg **(Figure 4 a).** In accordance with our hypothesis and *in vitro* data, pre-treatment with p19Arf/IFNβ

elevated the efficacy of the 10 mg/kg dose of Dox to the same level as the dose of 20 mg/kg, providing significant reduction in tumor progression **(Figure 4 a)** as well as substantial increase in survival, when compared to control groups GFP + PBS and Dox 10 mg/kg as monotherapy **(Figure 4 b)**. However, only those mice in the Dox 20 mg/kg group did not gain weight during the three weeks of therapy **(Figure 4 c)** and even more critically, as analyzed by echocardiogram, also developed a profound impairment of cardiac function. Indeed, after echocardiographic assessments, we revealed that cardiac function was compromised, as observed by left ventricular ejection function (LVEF) and left ventricular systolic function (LVSF) parameters **(Figures 4 d, e, f, and g)**. We also observed that ventricular mass was not altered by the treatment, which indicates that no aggravating anatomic changes were detected till the moment of the analysis **(Figure 4 h)**. Moreover, through the beating rate, we can attest that this analysis was performed in good standing patterns, as mice displayed similar beating rates per minute **(Figure 4 i)**. It is important to note that this analysis was performed 10 days after the i.t. treatment of Dox, representing an acute post-therapy event, which may be further aggravated over time.



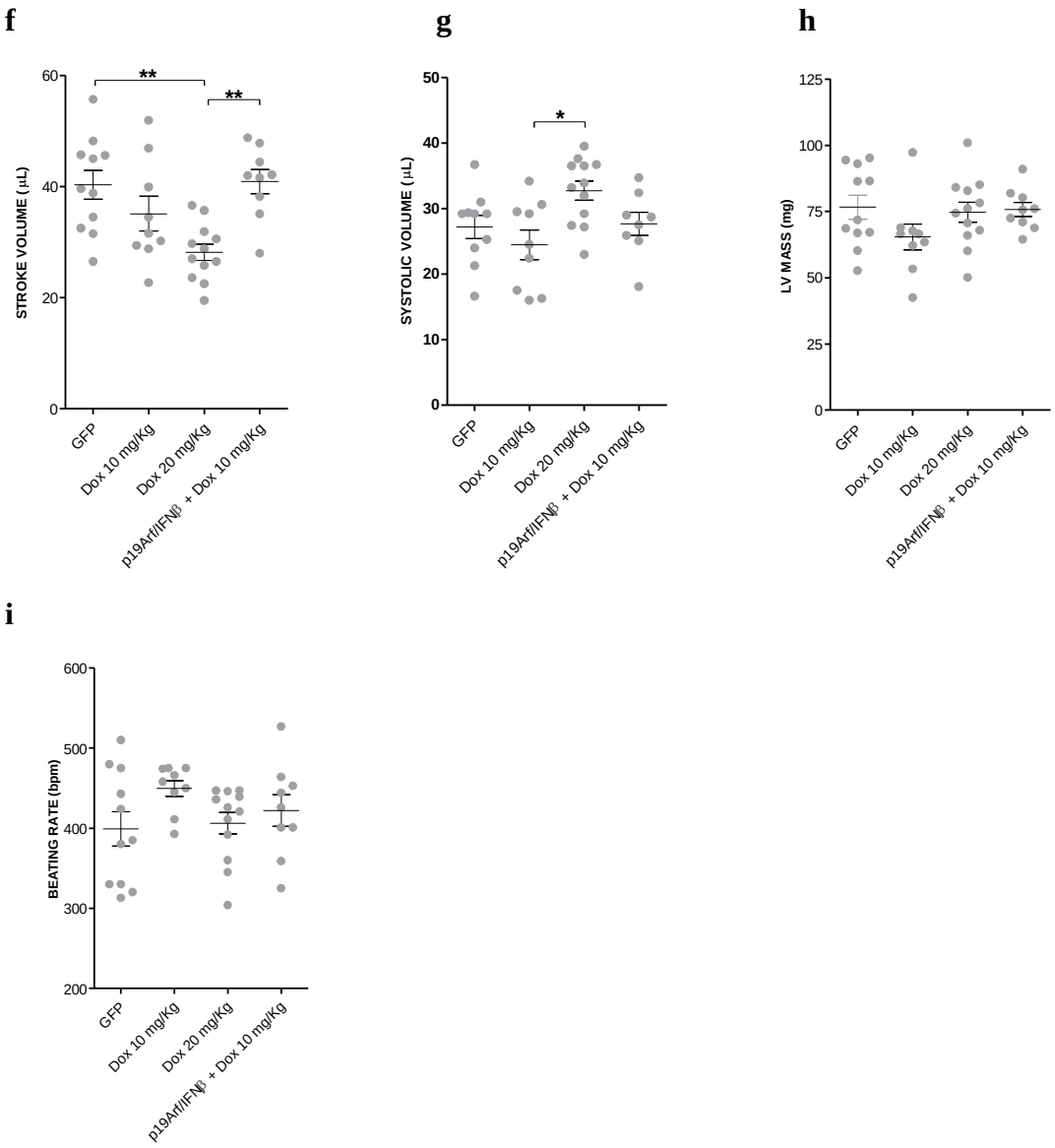


Figure 4. Pre-treatment with p19Arf/IFNβ gene therapy restores efficacy of sub-therapeutic dose of doxorubicin and maintains cardiac function (a) Tumor progression curves of established MCA tumors that were treated with 10 or 20 mg/kg of doxorubicin or in the association group, co-transduced with the AdRGD-PG-p19 and AdRGD-PG-IFNβ vectors and after 48 hours, also treated with 10 mg/kg of doxorubicin. [Two-way ANOVA and Bonferroni post-test] n=8 for all groups **(b)** Survival analysis of mice from a. [Log Rank Mantel-cox test, followed by Wilcoxon post-test]. **(c)** Monitoring of body weight during therapy [Two-way ANOVA and Bonferroni post-test]. Echocardiogram analysis 10 days after therapy of the **(d)** left ventricular ejection function, **(e)** the left ventricular systolic function, **(f)** stroke volume, **(g)** systolic volume, **(h)** left ventricular mass and **(i)** beating rate**.** n=9 for each group. [One-way ANOVA, followed by Tukey`s multiple comparison test post-test].

Impact of p19Arf/IFNβ and doxorubicin association on antitumor immunity

Another potential benefit of the p19Arf/IFNβ and Dox association would be to potentiate the antitumor immune response unleashed by each of these therapies. We hypothesize that by using two distinct ICD inducers, secretion of ICD DAMPS could be

modulated and result in increased immunogenicity of the treated cells. To address this hypothesis, we analyzed the exposition of calreticulin, which is *bona fide* ICD marker that acts as an "eat me" signal for professional APCs, by MCA and B16 cells after *in vitro* treatment. Although further experiments are needed to solidify this evidence, data gathered so far suggests an increase of CRT+/PI- and CRT+/PI+ cells upon *in vitro* treatment with p19Arf/IFNβ + Dox **(Figure 5 a)**. Other relevant ICD markers, such as ATP and HMGB-1, are currently being evaluated as well. To test if this combined treatment results in superior antitumor immunity, we developed a cancer therapeutic vaccine model, in which MCA or B16 cells were treated *ex vivo* with p19Arf/IFNβ, Dox or their combination, and before the start of the cell death process (i.e, not detectable by PI staining), injected (s.c) to die within the host and function as a cancer vaccine immunogen against a previously established growing tumor, termed as challenge tumor. Remarkably, only vaccination with p19Arf/IFNβ + Dox MCA cells reduced progression of challenge tumors, whereas cells treated with just p19Arf/IFNβ or Dox displayed little protection effect when compared to mice that received AdRGD-PG-eGFP transduced cells killed by freeze and thaw, a control expected to induce accidental necrosis and tumor antigen release **(Figure 5 b)**. However, it is important to note that mice vaccinated with cells treated with just p19Arf/IFNβ developed tumors at the vaccine site at late time points **(Figure 5 c),** which may be due to resistant clones already present in this cell line that, *in vitro*, were observed to repopulate the tissue plate after p19Arf/IFNβ therapy (data not shown). Therapeutic vaccination was also performed with B16 cells and once again p19Arf/IFNβ + Dox B16 cells profoundly inhibited progression of challenge tumors, as evidenced by 5 out 7 mice that fully rejected their tumors **(Figure 5 d)** and consequently presented survival superior to the monotherapies groups **(Figure 5 e)**. Although the immunological mechanism involved in this antitumor response needs to be investigated in more detail, these results provide strong evidence for the ability of the p19Arf/IFNβ + Dox association to augment immunogenicity of treated cells.

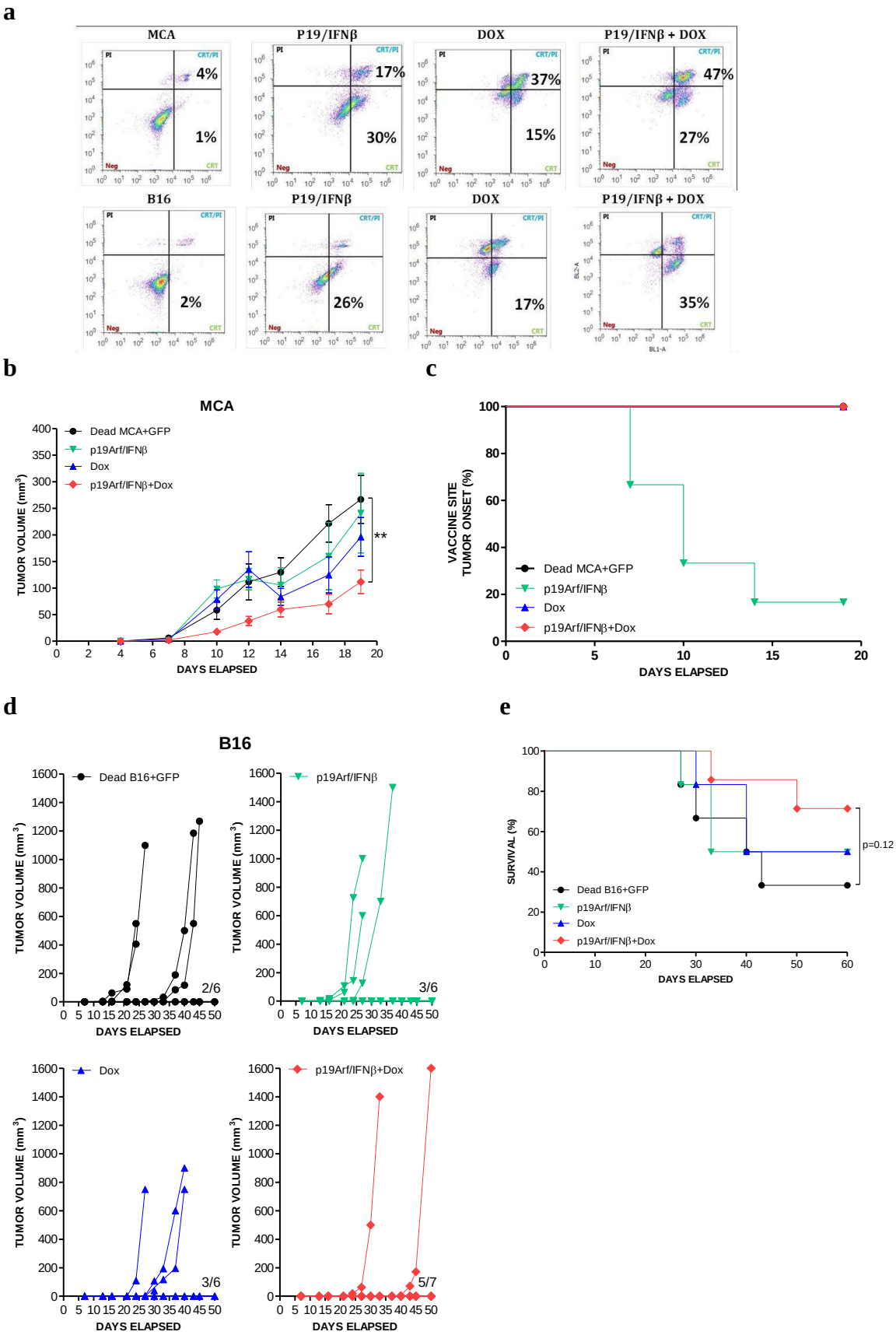


Figure 5. p19Arf/IFNβ and doxorubicin association potentiates immunogenicity of treated cancer cells. (a) Representative flow cytometry analysis of ecto-calreticulin exposure (CRT+ PI-) upon individual and combined treatment of MCA and B16 cells. n=2. **(b)** Therapeutic vaccine impact on the progression of challenge MCA tumors. [Two-way ANOVA and Bonferroni post-test] n=6 for all groups,

except for p19Arf/IFNβ + Dox where n=7. **(c)** Percentage of tumor-free mice at the vaccine site after inoculation of MCA cells transduced *ex vivo* with AdRGD-PG-p19Arf and AdRGD-PG-IFNβ vectors and/or treated with Dox**. (d)** Therapeutic vaccine impact on the progression of challenge B16 tumors. Data are presented as individual growth curves, n=6 for all groups, except for p19Arf/IFNβ + Dox that is n=7. **(e)** Survival analysis of the therapeutic vaccine from **d**. [Log Rank Mantel-cox test, followed by Wilcoxon post-test].

Translation of the phenomenon detailed in **Figure 5** to the *in situ* treatment model warrants experimental investigation since the immune suppressive microenvironment present within an established tumor can shape immunological events elicited by p19Arf/IFNβ + Dox treatment and different doses of Dox may have distinct effects on immunity. In order to formerly test this matter, we first investigated in both C57BL/6 and Nude mice if the association of p19Arf/IFNβ gene therapy with distinct doses of Dox (20, 10 and 5 mg/kg) provides comparable antitumor effects. As observed in **Figure 6 a**, association with any of the three doses inhibited tumor progression with a similar efficacy, having no statistical difference between them, and regardless of the dose, all were shown to depend on the immune system of the host. Since there seems to be a tendency for the association with the 20 mg/kg dose to increase survival more than with the dose of 5 mg/kg, this experiment is currently being repeated to increase statistical power **(Figure 6 b)**.

On the other hand, as revealed by the leucogram performed on peripheral blood collected on days three and five post-therapy, mice treated with just p19Arf/IFNβ presented markedly high number of blood circulating lymphocytes than the ones that were treated with AdRGD-PG-LUC, Dox 20 mg/kg and Dox 10 mg/kg **(Figure 6 c)**. Interestingly, this difference was not present when p19Arf/IFNβ was associated with Dox 10 mg/kg, suggesting that pre-treatment with p19Arf/IFNβ can alleviate immune suppressive side effects provoked by the use of high doses of Dox chemotherapy, as evidenced by the p19Arf/IFNβ + Dox 20 mg/kg group that showed a reduced number of lymphocytes. Although on day 5, p19Arf/IFNβ pre-treatment did not prevent reduction of cell number in any of the groups treated with Dox. And in accordance with the notion regarding high doses of Dox, transfer of splenocytes from mice that were treated (i.t) with 20 mg/kg of Dox and mixed with fresh MCA cells, displayed a similar protective effect as the ones treated with GFP vector, not affecting tumor progression **(Figure 6 d)**. Only mice that had their tumors treated with p19Arf/IFNβ as monotherapy presented significantly smaller tumors, yet this was compromised by the association with 20 mg/kg of Dox, arguing that high dose of Dox may negatively affect immunity previously elicited by p19Arf/IFNβ gene therapy. We consider this to be a critical question with important implications for our model, and we are currently performing a

secondary tumor challenge experiment, where mice carrying established MCA tumors are first treated p19Arf/IFNβ in association with different doses of Dox (5, 10 and 20 mg/kg) and subsequently challenged in the contralateral side with fresh MCA cells. Both progressions of the challenge tumor and infiltration of T cells are being monitored and compared to the monotherapy treatments.

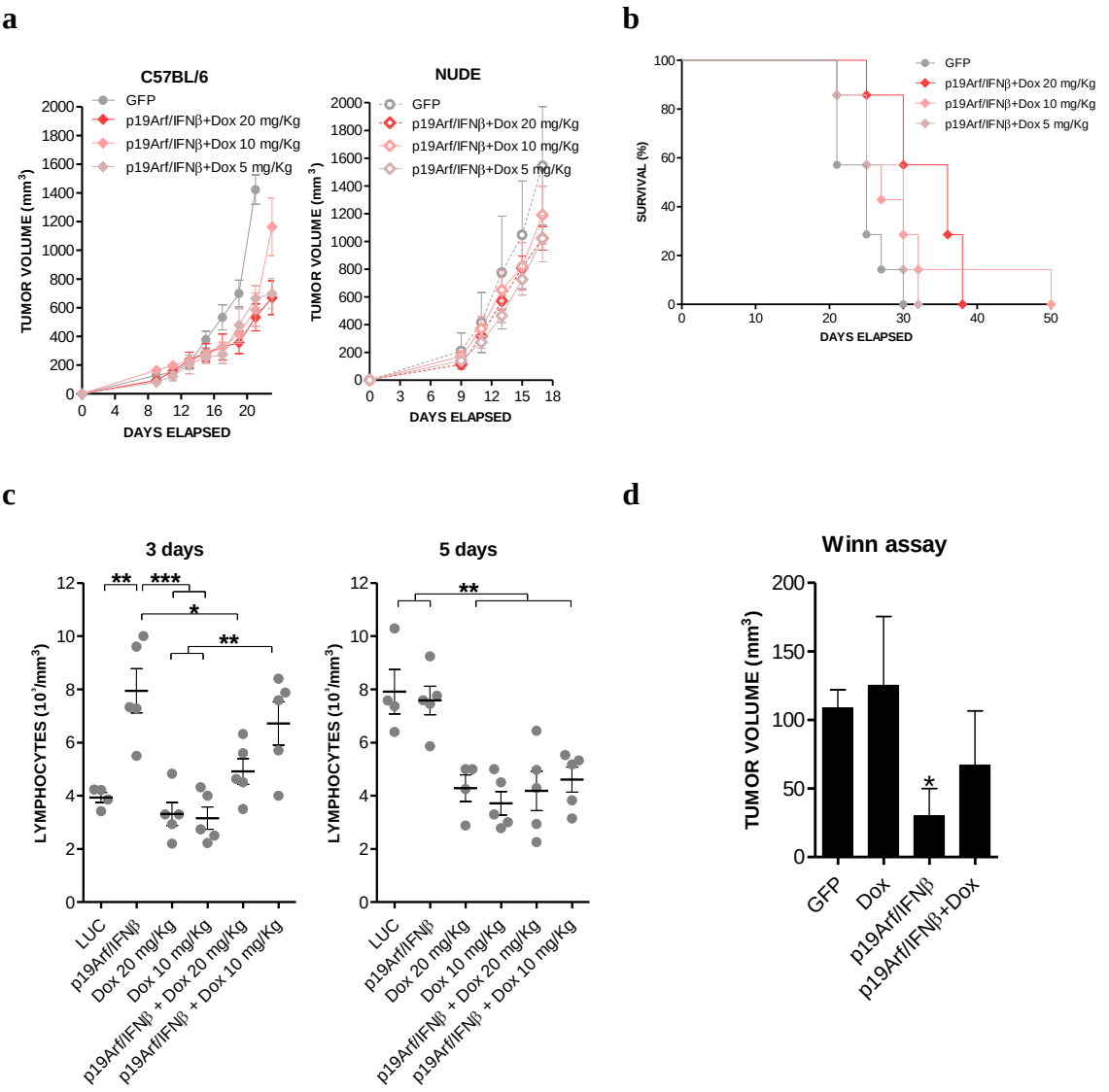


Figure 6. ***In situ*** **p19Arf/IFNβ and doxorubicin treatment affect host's immune system. (a)** Progression of MCA tumors in C57BL/6 and Nude mice upon *in situ* p19Arf/IFNβ gene therapy and Dox intratumoral treatment. n=6 for GFP (C57) groups and n=7 for all the C57 other groups. n= 2 for GFP (Nude) and n= 5 for the other Nuce groups. **(b)** Survival analysis of C57BL/6 mice from **a**. **(c)** Leucogram analysis of lymphocytes upon *in situ* individual or associated treatment with p19Arf/IFNβ and Dox. Peripheral blood collected on days 3 and 5 post-therapy. n=5 for all groups, except LUC where n=4. [One-way ANOVA and Tukey's multiple comparison post-test]. **(d)** Tumor volume 12 days after inoculation of splenocytes mixed with naive MCA cells. n=4 for all groups [One-way ANOVA and Tukey's multiple comparison post-Test].

p19Arf/IFNβ association with checkpoint blockade immunotherapy

In parallel with the attempts mentioned above, another hypothesis regarding the immunomodulatory properties of the p19Arf/IFNβ and Dox association is that upon

treatment, induction of inhibitory immune checkpoints, such as the CTLA-4 and PD-1 pathways, may occur and limit T cell immunity. Immune checkpoints are crucial for modulating duration and amplitude of immune responses in peripheral tissues (36) and considering the nature of using two ICD inducers together, that act by promoting inflammatory responses, it is tempting to speculate that these regulatory pathways are also being affected. Indeed, chronic expression of type I IFNs have been shown to favor maintenance of regulatory T cells, which are endowed with high levels of CTLA-4 expression, (38) and to induce the expression of PD-L1 in both tumor cells and immune infiltrates (39, 40). Along these lines, treatment with anthracyclines has also been shown to promote secretion of IFNβ by tumor cells in an autocrine regulated manner (41) and in theory, could also promote PD-L1 up-regulation. Thus, a triple therapy between p19Arf/IFNβ, Dox, and CTLA-4 or PD-1 blockade could have the capacity to unleash the full potential of the antitumor immune response.

In order to address this hypothesis, we first employed the B16 melanoma vaccination model without the association with Dox, since immunotherapeutic properties of our vectors are well described in this model and would, therefore, serve as a parameter for evaluation of future experiments involving the use of Dox. In the initial set of experiments, we tested CTLA-4 checkpoint blockade in the prophylactic vaccination model, where mice were immunized with B16 cells transduced *ex vivo* with AdRGD-PG-p19Arf and AdRGD-PG-IFNβ and 6 days after the vaccine, these mice were challenged with fresh B16 cells in the contralateral flank (day 0). In the association group, the CTLA-4 blockade was initiated 3 days post tumor challenge. Unexpectedly, association therapy in this prophylactic model had a strong antagonistic effect with the p19Arf/IFNβ vaccine, as tumors of this group displayed larger tumor volumes than those from the untreated group **(Figure 7 a)**. In the other groups, vaccination with p19Arf/IFNβ alone was more effective in reducing tumor progression than CTLA-4 blockade. Though the reasons for this antagonism are unknown, we hypothesized that by inhibiting the CTLA-4 pathway during the effector phase of the p19Arf/IFNβ immunotherapeutic response (initiated 6 days before tumor challenge) may have caused a deregulation of the immune attack, favoring tumor progression.

We again evaluate this association, but in a therapeutic model of vaccination, in which the p19Arf/IFNβ vaccine was performed 6 days after tumor challenge and during blockade of the CTLA-4 pathway (applied on day 3, 6 and 9). Interestingly, in this treatment regimen, the antagonistic effect reported above was no longer observed, but instead an additive effect was revealed, since tumor progression was drastically reduced

in the p19Arf/IFNβ + CTLA-4 group **(Figure 7 b)** and tumor development was completely inhibited in 2 out 6 animals, significantly increasing survival over the p19Arf/ IFNβ monotherapy **(Figure 7 c)**. In the group treated exclusively with CTLA-4, a reduction in tumor progression and an increase in survival were also observed, although less expressive than the associated therapy.

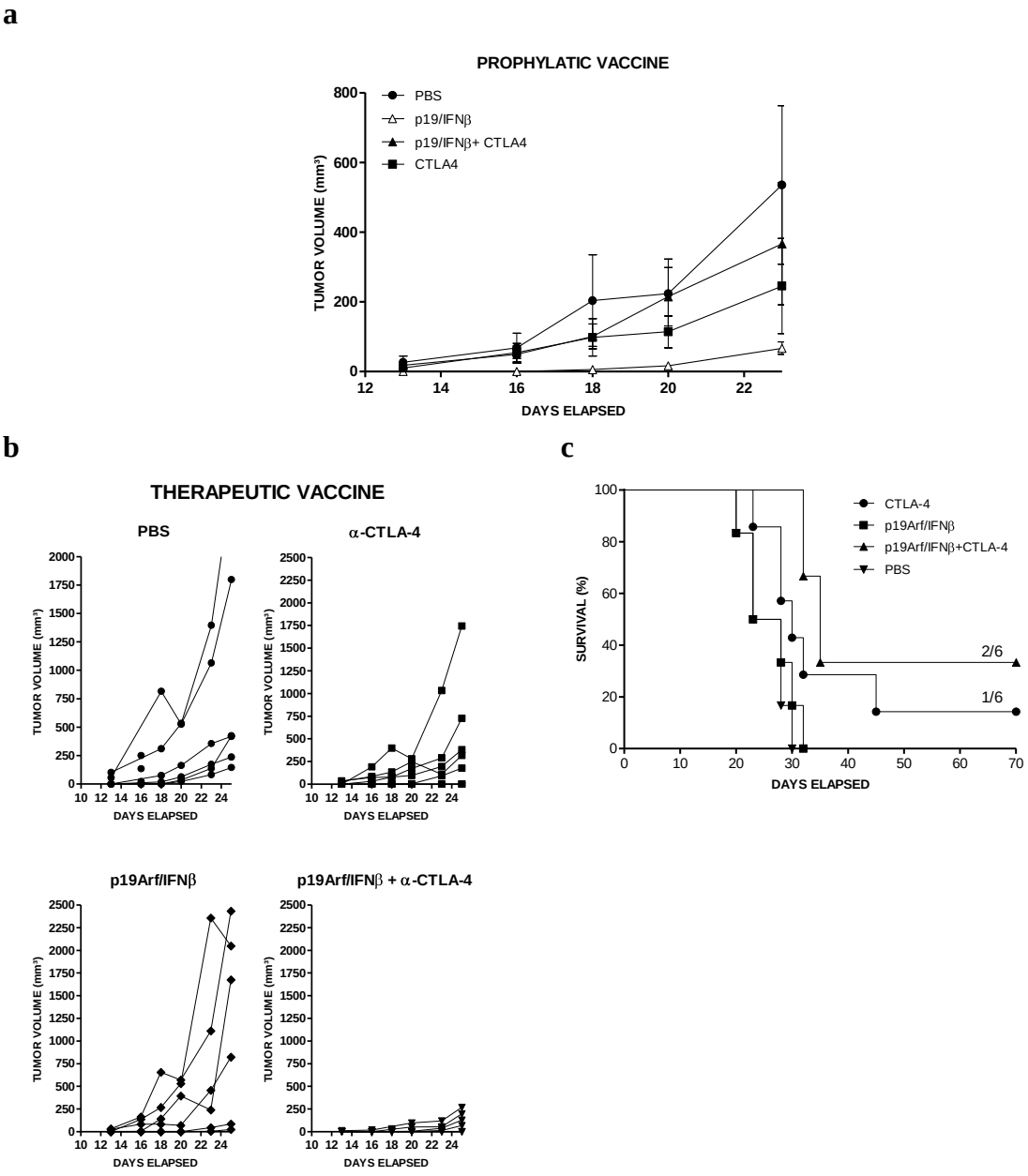


Figure 7. p19Arf/IFNβ vaccination combined with CTLA-4 blockade immunotherapy. (a) Progression of challenge tumors after prophylactic vaccination combined with CTLA-4 blockade. Mice were vaccinated on day -6 with B16 cells transduced *ex vivo* with AdRGD-PG-p19Arf and AdRGD-PG-IFNβ and after 6 days, on day 0, challenged with fresh B16 cells in the right flank. CTLA-4 blockade was performed on days +3, +6 And +9. n=6 for all groups. **(b)** Individual tumor progression curves from mice therapeutically vaccinated in combination with CTLA-4 blockade. Tumor challenge was applied on day 0 and p19Arf/IFNβ treated B16 cell applied as vaccine on day 6, during checkpoint blockade. n=6 for all groups. **(c)** Survival analysis of mice from b.

This result suggests that we can potentiate p19Arf/IFNβ immunotherapeutic effect by interfering in the regulatory mechanisms of the immune response. Even so, two optional strategies may be employed that may lead to more expressive results. In the first, therapeutic vaccination could be initiated on day +3, instead of day +6, since vaccine by itself was not effective on day 6. Data from the literature indicate that the therapeutic window for effective cancer control is small, and the later vaccination is initiated, the more difficult it is to obtain a protective effect (42). In the second, in view of the antagonistic result obtained with the prophylactic vaccine, we reason that, perhaps, the most appropriate association for an inflammatory vaccine such as ours is the blockade of the PD-1/PD-L1 pathway.

To evaluate these questions, the B16 therapeutic vaccine was repeated. However, the number of cells used in the tumor challenge was reduced (from $1x10^5$ to $6x10^4$ cells) to increase the therapeutic window, and the vaccination started on day 3 and repeated on day 10. Additionally, PD-1 and CTLA-4 blockade were also applied individually (on day 3, 6 and 9) as well as in association with p19Arf/IFNβ vaccine. As anticipated for this treatment schedule, p19Arf/IFNβ vaccine showed significant tumor control when applied by itself, reducing progression of challenge tumors in comparison to the Dead B16 + IgG Ab control group **(Figure 8)**. Of the two checkpoints, the performance of anti-CTLA-4 was superior to anti-PD-1, having more profoundly inhibited tumor growth. But, in opposition to the experiment **on Figure 7**, where p19Arf/IFNβ + CTLA-4 clearly blocked challenge tumor progression, here CTLA-4 worked better by itself, as its association with p19Arf/IFNβ seemed to provide no benefit, as no more than 2 animals per group were tumor free. The single use of anti-PD-1 seemed not to alter the tumor progression curves, yet when associated with p19Arf/IFNβ did we observe tumor rejection in 3/7 mice, a superior protective effect than seen with the p19Arf/IFNβ or PD-1 monotherapies as well as the CTLA-4 groups. More experiments are needed to confirm these results and use of the *in situ* treatment model may different outcomes, especially when adding Dox to the therapy, a question we are currently pursuing experimentally.

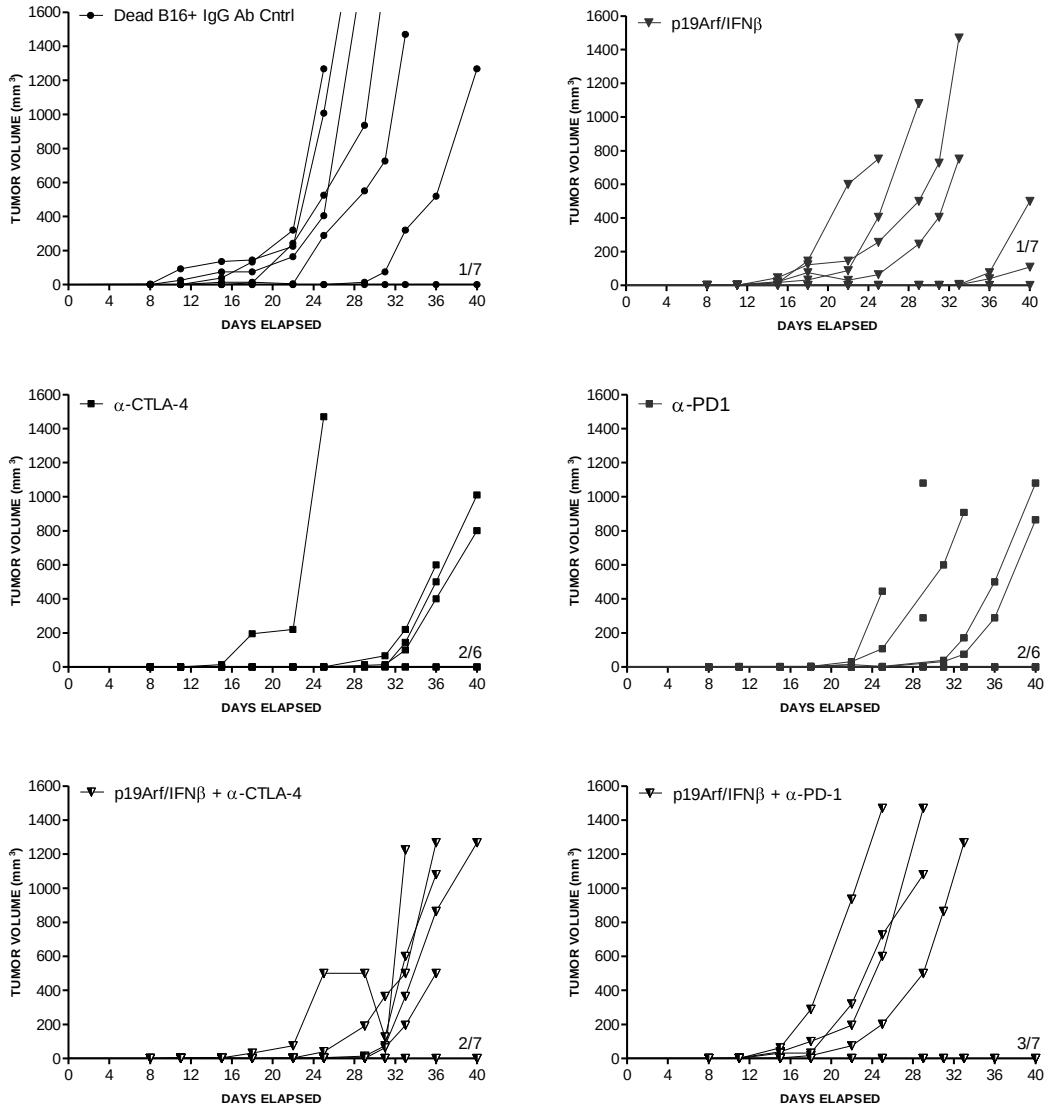


Figure 8. p19Arf/IFNβ therapeutic vaccine combined with CTLA-4 or PD-1 checkpoint blockade immunotherapy. Progression of individual challenge tumors after therapeutic vaccination in combination with CTLA-4 or PD-1 blockade. Mice were challenged on day 0 with fresh B16 cells and therapeutically vaccinated on days 3, 9 and 16 with B16 cells transduced *ex vivo* with AdRGD-PG-p19Arf and AdRGD-PG-IFNβ. Additionally, mice received CTLA-4 or PD-1 immunotherapy on days 3, 6 and 9. n=6 for all groups, except p19Arf/IFNβ + CTLA-4 and p19Arf/IFNβ + PD-1 (n=7).

DISCUSSION

In this study, we have explored potential benefits of applying p19Arf/IFNβ gene therapy in association with Dox chemotherapy, both of which elicit ICD, and as postulated here, their interplay could act on different levels of the therapeutic response. Previously, we have shown that reintroduction of $p19^{Arf}$ to cancer cells that harbor wild-type p53 leads to the activation of the p53 apoptotic program, but when an IFNβ antiviral context is induced simultaneously, an alternative mechanism of cell death is unleashed acting in a caspase 3 independent manner and through the up-regulation of

RIP3K, a critical mediator of necroptosis (20). Interestingly, Dox single treatment is known to activate p53, a point we have demonstrated previously (26) as well as in this current work, and here we show that Nutlin- 3, a compound able to specifically free p53 from its MDM2 mediated degradation (43), also activates expression from the PG promoter to a similar extent, indicating that the MCA cell line is endowed with transcriptionally functional p53 **(Figure 1 b)**. Yet, this result does not indicate that other functions of p53 are not altered, such as binding to the mitochondria, or that its ability to work as a transcription factor is not compromised or altered to some degree, or even that other members of the p53 family are also involved. Additionally, Dox is a well-known inducer of apoptosis and caspase 3/7 activity (17), as confirmed in **Figure 2 c**. Although not experimentally demonstrated here, transcriptional activation of p53 by the combined use of these agents could on theoretical grounds impact on the PG promoter and augment transgene expression levels, something previously explored with the combined use of $p19^{Arf}$ gene transfer and nutlin-3 drug treatment(44). This condition would be particularly interesting in the *in vivo* intratumoral treatment model, where, unlike *in vitro* transduction, we cannot assure delivery of a high MOI, so sub-optimally transduced cancer cells might experience insufficient transgene expression levels and may not activate molecular pathways involved in the p19Arf/IFNβ therapeutic effect. Thus, pre-treatment with the AdRGD-PG-p19 and AdRGD-PG-IFNβ vectors could be benefited by a later exposition to Dox that would start a novel round of PG expression along with cell death stimuli. Indeed, by the analysis of caspase 3 *in vivo* **(Figure 3 d)**, it seems this speculation may be justified since, in the p19Arf/IFNβ + Dox 24 H group, caspase 3 activity was diminished, just as in the p19Arf/IFNβ group. But upon Dox treatment, luciferase activity is then elevated at 48 hours. This suggests not only that cell death could be induced for longer periods, but also that Dox secondary treatment is acting on cells transduced previously and modulating their outcome. In fact, if this is the case, it may explain why unlike in **Figure 2 c**, that *in vitro* treatment with p19Arf/IFNβ + Dox provoked higher caspase 3 activity, *in vivo*, it was not superior or as high as Dox single treatment.

Although massive cell death may be needed to reduce tumor volume, induction of ICD can also evoke antitumor immunity and therefore, combinatorial use of distinct agents could provide different and additive stimuli, modulating the immunogenicity of treated cells. Indeed, as observed in **Figure 5**, by associating p19Arf/IFNβ + Dox, *ex vivo* treated cells provided superior antitumor protection in a therapeutic vaccine setting, what may be a result of up-regulating not only the already known ICD mediators but

also release of other molecules that have not yet been identified. Modulation of the immunogenic potential of cancer cells has already been demonstrated with cisplatin (CDDP), that as a single agent does not promote endoplasmic reticulum stress and therefore no translocation of calreticulin to the cell surface (45). But the association with thapsigargin, an inhibitor of the sarco/ER Ca(2+)-ATPase, endows CDDP with this ability and therefore a role in ICD. Thus, in future studies it will be interesting to investigate which of the ICD mediators are actually critical or even if high levels IFNβ provided by our vector can circumvent the lack of expression of one of them - a matter with important implications for cancer patients that present dysfunction in the ability to succumb to *bona fide* ICD, as already identified in breast cancer patients who carry a toll-like receptor 4 (TLR4) loss-of-function allele and consequently a defect in HMGB1 binding (16).

Additionally, modulation of immunogenicity should impact how dying cells interact with different APC subsets. For example, $CD169^+$ macrophages have been shown to dominate antitumor immunity by cross-presenting dead cell-associated antigens (46). But, induction of apoptotic ICD has been implicated to act on other APC subsets, since intratumoral $CD11c^+CD11b^+Ly6C^{hi}$ cells, which displayed some characteristics of inflammatory DCs and included granulomonocytic precursors, were observed to be vital for the immunogenicity of anthracyclines (47). Moreover, ATP released by dying cancer cells was also shown to recruit myeloid cells into the TME and promote differentiation of CD11c(+)CD11b(+)Ly6C(hi) cells. Expression of our AdRGD-PG-IFNβ vector should also bring about strong maturation and differentiation stimuli that are associated with IFNβ, possibly impacting on tumor-associated DCs, of which would be interesting to study the role of Baft3 DCs, which upon activation of the STING-IFNβ pathway can mediate recruitment of T cells with the TME through the CXCL9/CXCL10 axis (48, 49).

Yet, infliction of cell death can also negatively impact therapeutic outcome, as observed with localized radiotherapy that mediates caspase 3 activations and regulates prostaglandin E2 production, stimulating growth of surviving tumor cells and favoring tumor repopulation (30). Moreover, capture of apoptotic cell by $CD169^+$ macrophages has also been implicated in promoting rapid expression of the chemokine CCL22, inducing migration and activation of $FoxP3^+$ Tregs into the spleen, favoring classic apoptotic cell-induced immune suppression (50). Along these lines, as we have already mentioned, induction of inhibitory immune checkpoints could also come into play and hamper immunity. It is tempting to speculate that these mechanisms may explain why

the therapeutic effect from the association of p19Arf/IFNβ with the 20 mg/kg was not more pronounced as compared to the monotherapies **(Figure 3)**. It will be critical to evaluate levels of PD-L1 expression on tumor-associated CD45+ and CD45- cells upon p19Arf/IFNβ gene therapy, which besides revealing if cancer cells or immune host cells are actually mediating immune resistance, may also provide rationale for associating PD-1 checkpoint blockade – a immunotherapy proposed to work as foundation block for most combinatorial immunotherapeutic strategies (51), especially for those, such as the p19Arf/IFNβ + Dox association, that rely on promoting strong inflammatory immune responses.

Accordingly, a recent study from Pfirschke and collaborators has successfully exploited the association of immunogenic chemotherapy (oxaliplatin combined with cyclophosphamide) with anti-PD1 blockade (52). In this study, nonimmunogenic conditional genetic lung adenocarcinoma tumors that lacked infiltration of CD8+ T cells were treated with oxaliplatin combined with cyclophosphamide, rescuing T cell immunity and sensitizing tumors to CTLA-4 + PD-1 combined blockade. Additionally, these findings were extended to MCA tumors, which, on day 8, were treated (i.t) with 2.9 mg/kg of Dox and on days 8, 12 and 16 with CTLA-4 and PD-1 blockade, achieving superior tumor control.

Several other studies have also explored distinct intratumoral doses of Dox and reported its immunomodulatory properties (36, 47, 53-56). Yet, evidence indicates that the efficacy of each of these doses should be influenced not only by the tumor cell type, but also by the tumor size (57) or timing when treatment is applied, as evidenced by the dynamic changes in the immune infiltrate that correlate with tumor progression, where myeloid cells accumulate while the number of functional effector T cells is reduced (58, 59). Thus, despite the fact that here the 5 and 10 mg/kg doses were considered as sub-therapeutic, their use might have a different outcome if tested earlier during progression or in other cancer types. But then again, we believe that our model, where fully established tumors are treated, may better reflect the advanced stage at which most cancer patients are enrolled in the clinic. And it is in this scenario that association of an immunotherapy capable of promoting cell death and immune stimulation, such as ours, may offer an advantage over other strict immune modulators, specifically, sensitizing cancer cells to cell death and immune modulating the surrounding TME.

Our data related to the doses of Dox and their impact on immune stimulation or suppression is still inconclusive since the p19Arf/IFNβ + Dox associated therapy was ineffective when performed in Nude mice **(Figure 6 a)**, indicating that T cell immunity

is important for therapeutic control. Still, the leucogram analysis and Winn assay **(Figures 6 c and d)** suggest a dose-response effect that becomes antagonistic when the dose of 20 mg/kg was used. Even so, the interactions between p19Arf/IFNβ and Dox were shown to significantly potentiate induction of cell death, allowing the use of smaller doses of Dox as well virus MOI **(Figure 2 b and c).** Accordingly, association with p19Arf/IFNβ provided notable benefit for the 10 mg/kg dose of Dox, providing a therapeutic effect comparable to the 20 mg/kg dose, yet preserved cardiac function **(Figure 4)**, a major potential advantage in the clinical cancer setting. Thus, in conclusion, the evidence presented here unveiled relevant therapeutic benefits of using the $p19^{Arf}$ and IFNβ gene therapy in association with Dox, a standard of care immunogenic chemotherapeutic agent. And also, this study paved the way for other combinatorial approaches, such as with checkpoint blockade immunotherapy, that hold great potential for the further improvement of therapeutic efficacy.

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AUTHOR CONTRIBUTIONS

RFVM and BES conceived and designed the experiments; RFVM analyzed the data, wrote the manuscript and performed the experiments in Figures 1, 2, 3, 4, 5, 6, 7 and 8. RET performed the experiment on figure 6 a and b. SAM assisted with the experiments on Figure 4 and provided the AdRGD-PG-LUC vector. VAF performed the central composite rotational design analysis on Figure 2 b. TAS helped to conceive the echocardiogram analysis and helped on the experiment in Figure 4. RD helped to conceive the echocardiogram analysis and performed the cardiac function analysis in Figure 4. AHR provided help with calreticulin assay and viral stocks for production. JPPC helped with the winn assay on figure 6 d. EGR discussed the data and participated in the experiment planning. BES discussed the data and supervised the study.

8

General discussion and conclusion

GENERAL DISCUSSION

Recent advances in cancer immunotherapy have changed the landscape of cancer treatment. And as the field is experiencing unprecedented momentum, our knowledge of the determinants that are involved in an antitumor immune response have greatly increased, what in contrast to past approaches, allows modern strategies to enter in the fight against cancer with "eyes wide open", or even perhaps, attempting to anticipate cancer's next move, a topic presented in **Chapter 2**.

Given this current perspective of the field that, we will discuss in this last chapter the opportunities and challenges of harnessing the cooperation between the p53/Arf and interferon-beta (IFN-β) pathways as a mediator of cancer immunotherapy.

The $p19^{Arf}$ and IFN-β immunity cycle

Based on the studies presented in **Chapter 4, 5 and 6,** we propose the following model to describe the immunity cycle initiated upon the combined gene transfer of $p19^{Arf}$ and IFN-β to cancer cells **(Figure 1)**: *first*, either in the vaccine or in the *in situ* gene therapy model, the host immunization happens as a consequence of the presence of treated cancer cells, in which the AdRGD-PG-p19 vector promotes the reestablishment of the p53/Arf pro-apoptotic pathway (evidenced by up regulation of caspase-3 activity, Bax and other p53 target genes), which by itself is not strong enough to unleash massive cell death levels. But along with the delivery of the AdRGD-PG-IFNβ vector, an endogenous antiviral defense program is also activated, alternating the apoptotic process to an immunogenic cell death (ICD) process that displays features of necroptosis, as indicated by the up regulation of RIP3K, mediator of the necroptossome complex (1). Together with these events, $p19^{Arf}$ and IFN-β combined gene transfer promotes the expression of key natural killer (NK) cell activators, interleukin-15 (IL-15) and UL16 Binding Protein 1 (ULBP1), along with the death receptors FAS/APO-1 and KILLER/DR5, which collectively should impact the cytotoxic activity of these innate immune cells and result in the rejection of transduced cells at the vaccine site. *Second*, in response to inflammatory mediators released by dying cells and the up-regulation of CCL3, CXCL3 chemokines as well as IL-1β, within the tumor mass, $CD11b^{+}$ $Ly6G^{+}$ neutrophils are recruited and display a critical antitumor role, as suggested by the antibody-mediated granulocyte depletion that negatively affected therapy outcome. Although the exact function exerted by this neutrophils remains to be elucidated, they may participate by reshaping the tumor tissue after damage and/or by favoring infiltration of $CD8^{+}$ T cells, which were also indicated to be involved by transcriptome analysis of *in situ* treated tumors (2). *Third*, secretion of high levels of IFN-β acts

directly on transduced cells and also in bystander cells, modulating the surrounding tumor microenvironment, which together with the release of ICD molecules (i.e., calreticulin, ATP and HMGB-1), are proposed to strongly affect maturation and function of tumor associated antigen presenting cells (APCs), that mediates effective priming of T cells and promotes a Th1 immune response, revealed by the detection of IFN-γ and tumor necrosis factor (TNF-α). *Lastly*, induction of $CD4^+$ and $CD8^+$ T cells promotes antitumor protection mostly at distant or secondary tumor sites and provides long term immunity (3).

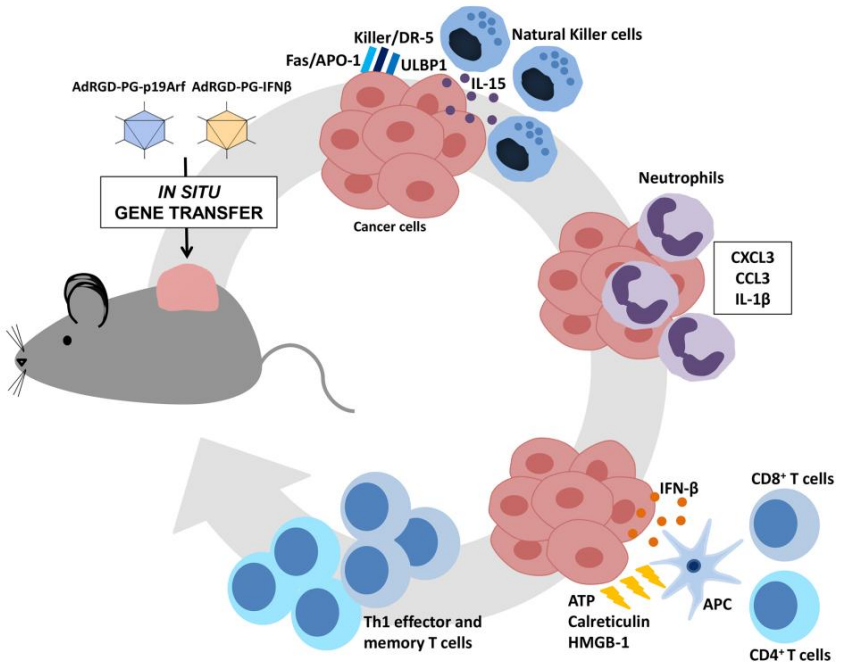

Figure 1. Working hypothesis on the $p19^{Arf}$ and interferon-β immunity cycle. Upon *in situ* gene transfer with the AdRGD-PG-p19 and AdRGD-PG-IFNβ adenoviral vectors, cancer cells succumb to a necroptotic immunogenic cell death (ICD) process that, through the up- regulation of interleukin-15 (IL-15), UL16 Binding Protein 1(ULBP1) and the death receptors FAS/APO-1 and KILLER/DR5, promotes the engagement of natural killer (NK) cells. Subsequently, within the tumor mass, up-regulation of CCL3, CXCL3 chemokines as well as IL-1β, orchestrate the recruitment of $CD11b^+$ $Ly6G^+$ neutrophils, which are thought to act by favoring infiltration of $CD8^+$ T cells. Next, secretion of interferon-β (IFN-β) together with the release of ICD molecules calreticulin, ATP and HMGB-1, augments T cell priming ability of tumor associated antigen presenting cells (APCs), driving a Th1 immune response, evidenced by secretion of IFN-γ and tumor necrosis factor (TNF-α). Finally, mediated by $CD4^+$ and $CD8^+$ T cells, antitumor protection combats distant or secondary tumor sites and provides long term immunity (3).

On the basis of this evidence, we consider that we have amassed sufficient functional demonstrations to allow us to characterize the combined gene transfer of $p19^{Arf}$ and IFN-β (p19Arf+IFN-β) as a cancer immunotherapy strategy. To the best of our knowledge, this is the first attempt to exploit the cooperation between these

pathways as means to treat cancer. In fact, no other gene transfer method employing replication defective vectors has been reported to be mediating the release of classic ICD, have received FDA approval, adding to the novelty of our approach in the gene therapy field.

We acknowledge that several important questions remain open. For example, tumor onset in the vaccine site was abrogated in hosts with functional NK cells, though detection of these cells was not performed and neither was a proper analysis of NK activation status upon encounter with B16F10 (B16) cells treated with p19Arf+IFN-β or with just IFN-β. Mounting evidence in the literature indicates that a subset of NK with a helper phenotype can influence the ability of dendritic cells (DCs) to prime T cells (3), a function that may be revealed upon NK cell depletion during immunization and/or effector steps of the response. Additionally, neutrophils can also impact the recruitment, activation and effector function of T cells, mechanisms that remain to be elucidated in our model (2). Superior immunogenicity of p19Arf+IFN-β treated cells should also result from the combined impact that IFN-β and ICD molecules can exert on tumor associated APCs, of which $CD169^{+}$ macrophages (4, 5), $CD11c^{+}CD11b^{+}Ly6C^{hi}$ (6) and Baft3 DCs (7, 8) are potential subsets to be investigated. Most notably, use of knockout (KO) cancer cells for calreticulin and HMGB-1 might reveal if there is an additive effect of these molecules when in the presence of IFN-β, or even if IFN-β can circumvent the lack of one of them.

Challenges and opportunities within the $p19^{Arf}$ and IFN-β immunity cycle

Aiming to anticipate cancer's next move, that is to say, how malignant cells might inhibit or take advantage of cell death and inflammatory stimuli induced by the p19Arf+IFN-β gene transfer, we present potential therapeutic challenges while making a case for how these challenges could be overcome by associating other therapies in a rational manner **(Figure 2).**

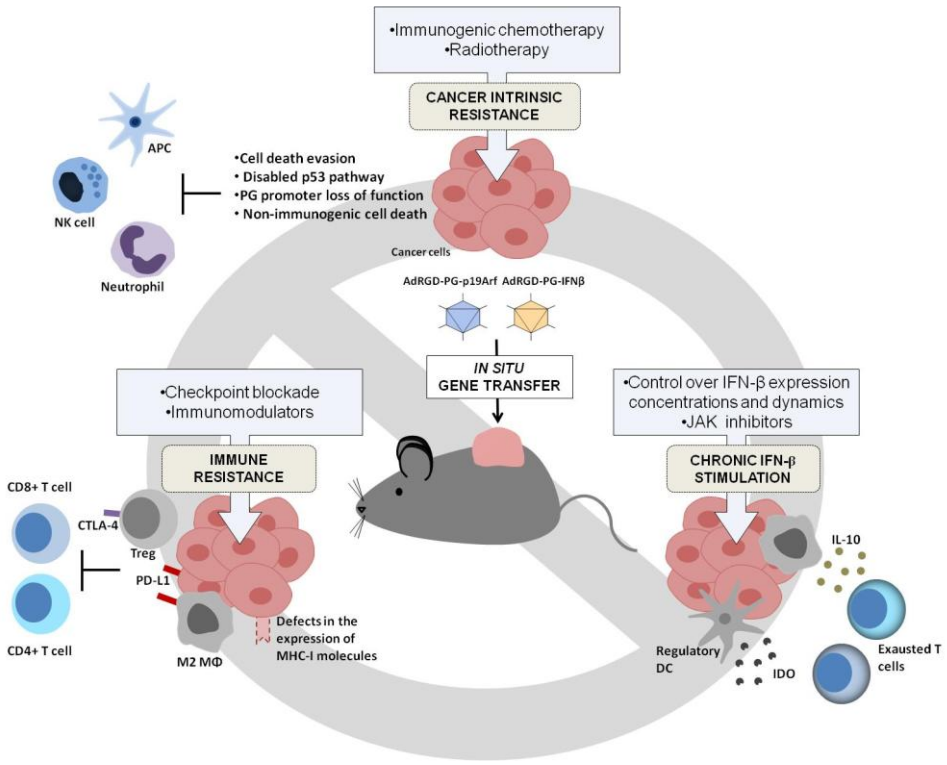

Figure 2. Proposed challenges and combinatorial opportunities within the p19^{Arf} and interferon-β immunity cycle. Along with antitumor events unleashed by the *in situ* gene transfer utilizing the AdRGD-PG-p19 and AdRGD-PG-IFNβ adenoviral vectors, multiple counteracting resistance mechanisms (represented in the light green rectangles) are also thought to take place and hamper therapeutic efficacy. To start, induction of the apoptotic p53 program could exert selective pressure and lead to cell death evasion, which, by disabling function of p53 and its partners, would contribute to loss of function of the PG promoter, blunt induction of immunogenic cell death (ICD) and fail to stimulate innate compartments, such as natural killer (NK) cells and dendritic cells (DCs). Overcoming these aspects of cancer's intrinsic resistance scenario could be achieved by augmenting cell death stimuli and immunogenicity of treated cells through the association of immunogenic chemotherapy, such as doxorubicin, or radiotherapy (represented in light blue retangles). Additionally, chronic and unregulated production of interferon-β (IFN-β) have been shown to induce production of interlukin-10 (IL-10) by macrophages (mΦ), indoleamine 2,3-dioxygenase (IDO) enzyme by regulatory DCs and consequently, T cell exhaustion. Induction of this IFN-β regulatory mechanism could be reverted by controlling expression dynamics of IFN-β or by the use of janus kinases (JAK) inhibitors, which would interfere with the JAK-STAT signaling pathway and avoid STAT driven inhibitory programs. Alternatively, effector function of $CD4^+$ and $CD8^+$ T cells could be inhibited by the programmed death-ligand 1 (PD-L1) program expressed on both mΦ and cancer cells, or alternatively, by regulatory T cells (Treg) that are endowed with high levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4). Along these lines, immune therapeutic pressure has been shown to cause different defects in MHC class I antigen presentation, leading to immune evasion of cancer cells. Yet, association with checkpoint blockade immunotherapy or other immunomodulators is expected to recover antitumor immunity.

The theoretical challenges underlying the use of p19Arf/IFN-β as cancer immunotherapy may be encountered at distinct a level, which does not mean an actual order of events, that a level is superior to the other or even that cooperation between them is not possible. For example, it is well known that cancer's intrinsic resistance to cell death stimuli arises as a result from its capacity to circumvent the pressure exerted by the p53 apoptotic program (9), which in response to its pivotal role in protecting against malignant transformation, the p53 protein is found to be mutated in more than

50% of cancers while the cancers that retain a normal *TP53* gene present mutations in genes related its function (10). This scenario suggests that either p19Arf+IFN-β treatment will most likely will be met with an alteration present prior therapy or that the selection pressure created by therapy bring defects in the p53 pathway into play, impacting not only cellular mechanisms but also the PG promoter. Even so, the alteration of the cell death process from apoptosis to necroptosis observed only upon combined gene transfer could represent an advantage in this scenario by assuring cell death induction by one pathway or the other. Indeed, knockdown of genes highly expressed in response to p19Arf/IFN-β still resulted in the effective induction of cell death (11), suggesting that either we have not yet found the critical gene or that there may be extensive plasticity underlying the cell death pathway. On this note, even if p53 itself is found to be mutated, microarray analysis indicates that other family members are also greatly impacted by p19Arf+IFN-β treatment - especially p73, whose transcript was shown to be more than 1000 times higher in response to this combined gene transfer (11). Additionally, as shown in **Chapter 7**, combinatorial use of chemotherapeutic agents such as doxorubicin (Dox) or other ICD inducers, such as oxaliplatin and radiotherapy, could be useful in reverting or avoiding this resistance scenario, massively potentiating cell death and targeting ICD by multiple pathways.

Immunomodulatory properties of both IFN-β and IFN-γ could also be considered as critical targets for intrinsic resistance, as already reported for different immunotherapy strategies that somatic mutations can endow cancer cells with the ability to inhibit the IFN signaling pathways (12). For example, chromosomal alterations and JAK1 or JAK2 inactivating mutations were recently shown to shield melanoma cells from IFN-γ activity and to develop into T-cell-resistant HLA class I-negative lesions with silencing of genes involved in antigen presentation (13). These and other (14-16) resistance mechanisms related to IFNs may arise as a consequence of immune pressure and cancer's intrinsic adaptability, but may be further aggravated by the treatment regime as well, which if designed without carefully considering this possibility might end up favoring resistance. Indeed, both timing and magnitude of type I IFN stimulation have been demonstrated to have important consequences on the regulation of the response (17, 18) and this a key question for future studies of our group, since up until now, our immunotherapy applied as *in situ* gene therapy has employed sequential injections with the adenoviral vectors at 48-hour intervals, with(three to six total treatments injections. We have not yet evaluated proportions between vectors, if prolonged time between injections could extend the effect of

controlling tumor progression, or even, if upon use of more injections could result in augmentation or down regulation of the response. In fact, it remains elusive why the *in situ* gene transfer of IFN-β alone results in similar tumor regression as seen when combined with $p19^{Arf}$, that promotes cell death. One possible consideration is that anti-angiogenic effects exerted by IFN-β may be overcoming cell death and antitumor immunity, as demonstrated in the work of Spaapen and colleagues where intratumoral delivery of high levels of IFN-β acts primarily by inhibiting tumor angiogenesis rather than on the host immune cells (19). Experiments with cell lines lacking the type I IFN receptor (IFNAR1) or use of IFNAR 1 K.O mice, that are not sensitive to type I IFNs, might reveal the importance of signaling in the tumor cell versus the host.

Adding to the complexity of this issue, as proposed in **Chapter 3**, type I IFNs have a context dependent and complex role in regulating cancer immunity and their use would be benefited by more sophisticated methods of delivery that assure more localized concentrations along with a tighter control over expression dynamics, avoiding immune suppressive mechanisms mostly observed under conditions where type I IFNs are chronically produced. These regulatory effects function by inducing production of IL-10 by macrophages, tryptophan starvation of regulatory DCs mediated by indoleamine 2,3-dioxygenase (IDO), maintenance of FoxP3 transcriptional factor in regulatory T cells (Tregs) (20). Most notably, both type I and II IFNs are strong inducers of the programmed death-ligand 1 (PD-L1) on a myriad of different cells, including macrophages (21) and melanoma cells (22). And so, as postulated on **Chapter 7**, association of PD-1 checkpoint blockade holds great potential to augment therapeutic efficacy of our vectors, especially in the *in situ* treatment model. Although, combined use with IDO inhibitors (23), Treg depletion (24) or NK cell modulators (25) are also tempting notions to be investigated.

The requirement of type I IFNs for maintaining a PD-L1 independent immunotherapy resistance phenotype was also recently implicated, surprisingly, provoked by a Signal Transducer and Activator of Transcription-1 (STAT-1) epigenetic signature and as such, could be successfully reverted by the use of janus kinase (JAK) 1/2 inhibitors, sensitizing previously resistant tumors to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) checkpoint blockade (26). If an inhibitory STAT-1 or STAT-3 signature were to be observed in our model, which the latter of is better known for attenuating the inflammatory properties of type I IFNs, then association of JAK inhibitors such as the ones used in the mentioned study, may provide a means to exploit the therapeutically beneficial side of IFN-β. The fact that AdRGD-PG-p19 and

AdRGD-PG-IFNβ co-transduced cells succumb to cell death, as opposed to cells treated with just AdRGD-PG-IFNβ, may offer another opportunity to exploit the dynamics of transgene expression. Expression would be terminated upon cell death, though treatment with IFNβ alone should yield the transgene product over longer periods of time and possibly contribute to the induction of regulatory mechanisms that limit the response. Use of IFN-β vectors also carrying a luciferase gene reporter may help us to reveal this difference in expression kinetics *in vivo*.

Although our approach described in **Chapter 7** a more detailed analysis of the immune response, the vaccine model in association with Dox or checkpoint blockade immunotherapy indicates that combinatorial approaches can indeed augment immunogenicity of p19Arf+IFN-β treated cells. Future studies should aim to identify the major inhibitory mechanisms at play in our model, as these may offer the opportunity to turn challenges into advantages, unleashing the full potential of immunity against cancer cells.

Translational perspectives of using $p19^{Arf}$ and IFN-β as a cancer immunotherapy strategy

So far in this discussion we have focused on evidence gathered in mouse models of cancer, but if we were to consider the next steps in therapeutic development, it is also important to examine the translational perspective. And towards that end, the first step would be to develop adenoviral vectors that encode the human transgenes of $p19^{Arf}$ ($p14^{ARF}$ in humans) and IFN-β and aim to recapitulate in human melanoma cell lines the cell death molecular events observed in the murine setting. In fact, our lab has already begun to explore this step, where the AdRGD-PGhIb vector that encodes the human cDNA of IFN-β used to transduce the SK-MEL-05 and SK-MEL-147 melanoma cell lines leads to a robust cell death response. But, its association with a vector encoding the $p14^{ARF}$ cDNA has not been tested yet (27).

Future development should also contemplate the exact role of p53 and its family members since, as already mentioned, p53 is needed to drive expression of the vector and also participates in the therapeutic effect. Although the use of a vector with a constitutive promoter, such as CMV, may at first seem to be an ideal alternative to treat cell lines harboring mutated p53, in a previous study of our group the PG system was also used to deliver the p53 cDNA to prostate cancer cells and resulted in superior killing than a typical AdCMVp53 vector (28). In part this result may reflect the high expression levels achieved the PG system, but also the ability of the PG vector to resonate with the dynamics of p53 expression, shown to be critical for dictating which

set of genes will be targeted by p53 (29), an effect that could not be achieved with a standard p53 vector. Along these lines, the impact of different mutant p53 proteins, which may lose wild-type p53 tumor suppressor activity and gain pro-tumoral functions, should also be investigated (30), aiming to identify a proper genotype that would favor the gene transfer of $p14^{ARF}$ and IFN-β. At least in theory, use of a tri-cistronic adenoviral vector, encoding p53, $p14^{ARF}$ and IFN-β may assure that all three players are properly delivered, maximizing transgene function despite the cancer genotype.

Having said that, an obvious question that comes to mind is regarding which tumors could we actually treat.

Beyond the p53 genotype, the determination of whether tumors arising from different tissues or carcinogenic pathways will also be susceptible to our approach represents an obvious, though unanswered, question. On this note, as mentioned in **Chapter 1**, melanoma tumors represent an ideal target, as this cancer harbors wild type p53 in more than 90% of the cases (31) and displays high immunogenicity (32). But, as seen in mouse models, the LLC lung carcinoma and MCA sarcoma cells lines could be successfully treated as well, the latter of which, interestingly, was induced by the use of the methylcholanthrene carcinogen that inflicts DNA damage similar to the use of tobacco (33).

Although it is still early for this consideration, one last aspect to be discussed in terms of translational research would be designing the treatment modality for the application of our vectors. Only a few clinical protocols using recombinant vectors for the delivery of IFN-β in cancer patients have been carried out (34-36), indicating that intratumoral injections are generally safe and potentially effective (37), but also that there is room for improvement, a conclusion that supports future clinical investigation of our vector. Another interesting therapeutic application would be the use of p19Arf+IFN-β treated cancer cells to provide, through the induction of ICD and IFN-β release, adjuvant stimulation as well as tumor antigens for *ex vivo* derived DC vaccines, an application that was recently demonstrated in a pre-clinical model of high-grade glioma tumors (38). Nonetheless, despite the long road ahead, we consider that the perspectives of exploiting the combined gene transfer of $p19^{Arf}$ and IFN-β as cancer immunotherapy strategy warrants future development.

CONCLUSION

In an endeavor to answer the central question that generated this thesis, whether the cooperation between the p53/Arf and interferon-β pathways could be applied as a cancer immunotherapy strategy, we have explored several aspects of both *in situ* gene therapy and vaccine models and revealed that the cell death unleashed by the adenoviral mediated gene transfer of $p19^{Arf}$ together with interferon-β works as mediator of antitumor immunity. Indeed, we have identified natural killer cells, $CD4^+$ and $CD8^+$ T lymphocytes, neutrophils and immunogenic cell death molecules to be key players of our therapy.

Additionally, employing doxorubicin and checkpoint blockade immunotherapy, we also touched upon on combinatorial strategies that have the potential to further extend immunotherapeutic benefit. Most importantly, we have raised new questions and hypotheses that could point the way for future translational applications of our adenoviral vectors and, hopefully, contribute to the treatment of cancer.

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Para que as células do câncer possam se desenvolver, elas precisam aprender a resistir aos estímulos que lhes matariam e também a se esconder do sistema imunológico. Nós propomos que podemos reestabelecer a capacidade do organismo de combater o câncer através da terapia gênica, em que utilizamos em combinação vírus geneticamente modificados para levar um gene causador de morte, chamado de p19Arf, e outro gene estimulador da resposta imune, chamado de interferon-β. De fato em nossas primeiras observações, notamos que células de melanoma de camundongo morrem mais quando tratadas com estes dois vírus. Porém como a capacidade desta combinação de ativar uma resposta imunológica não foi analisada previamente, o objetivo desta tese foi investigar, em modelos experimentais, se o uso de células tumorais tratadas com estes vírus poderia imunizar camundongos e assim, funcionar como vacina e proteger contra o desenvolvimento de tumores. Os resultados obtidos indicam que células de melanoma mortas pelo tratamento com p19Arf e interferon-β ativam diferentes tipos de células imunes, entre elas os linfócitos T, que agem para diminuir o ritmo de crescimento tumoral. Por fim, buscando melhorar ainda mais esta proteção imunológica, estudamos a associação dos vírus com o quimioterápico chamado doxorrubicina e com anticorpos que controlam a atividade dos linfócitos T, chamados de anti-PD1 ou de anti-CTLA4. E neste cenário, a associação destas terapias provocou a eliminação de tumores em um maior número de animais. Sendo assim, as evidências apresentadas aqui indicam que os nossos vírus geneticamente modificados tem o potencial de ser desenvolvidos como uma terapia baseada na ativação da resposta imunológica, ou seja, uma estratégia de imunoterapia do câncer.

In order for cancer cells to develop, they need to learn to resist stimuli that would kill them and also, to hide from the immune system. We propose that we can reestablish the body's ability to fight cancer through gene therapy, in which we use in combination genetically modified viruses to carry a gene that causes death, called p19Arf, and another immune-stimulating gene, called interferon-β. In fact, in our first observations, we noticed that mouse melanoma cells die a lot more when treated with these two genes together. However, since the ability of this combination to activate an antitumor immune response has not been previously analyzed, the aim of this thesis was to investigate, in experimental models, whether the use of tumor cells treated with these viruses could immunize mice and thus function as a vaccine and protect against development of tumors. Our results indicate that melanoma cells killed by the treatment with p19Arf and interferon-β activates different types of immune cells, including T lymphocytes, which act to decrease the rate of tumor growth. Finally, in order to further improve this immunological protection, we studied the association of the viruses with the chemotherapeutic drug called doxorubicin and with antibodies that control the activity of T lymphocytes, called anti-PD1 or anti-CTLA4. And in this scenario, the association of these therapies provoked the elimination of tumors in a greater number of animals. Thus, the evidence presented here indicates that our genetically modified viruses have the potential to be developed as a therapy based on the activation of the immune response, or in another words, a cancer immunotherapy strategy.

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News and Commentary

Harnessing combined p19Arf and interferon-beta gene transfer as an inducer of immunogenic cell death and mediator of cancer immunotherapy

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Cancer immunotherapy is a wide-ranging term that includes many strategies to reestablish or activate an effector antitumor immunity cycle. Among the most successful approaches, PD-1/PDL-1 and CTLA-4 checkpoint blockade and chimeric antigen receptor (CAR) T cells have provided undeniable evidence of the potential of immunotherapy for several types of cancer, including metastatic melanoma.[1]

Another promising approach that aims to provide both antigenic and adjuvant signals in order to initiate an adaptive immune response is the induction of immunogenic cell death (ICD). It was originally described as a molecular response of cancer cells to treatment with chemotherapeutic agents (e.g., anthracyclines), and involves the orchestrated release of the danger signals calreticulin, ATP and HMGB-1.[2] Interestingly, this cell death process can be triggered by viral infections and is one of the mechanisms by which some oncolytic viruses, such as those based on herpes simplex virus type 1 (HSV-1), induce an immune response.[3] However, to the best of our knowledge, gene transfer methods mediated by nonreplicative viral vectors, such as adenovirus, have yet not been described as inducers of ICD.

With the objective of reestablishing both intrinsic cell death mechanisms and cancer immune surveillance, our lab developed a unique set of adenoviral vectors for the gene transfer of both p19Arf (functional partner of p53) and interferon-β (IFNβ, immunomodulatory cytokine). As elucidated in our recent work,[4] targeting the p19Arf and IFNβ pathways created interplay between (i) p53/Arf pro-apoptotic signaling, (ii) the adenovirus delivery vehicle and (iii) an IFNβ antiviral/immunostimulatory pathway, culminating in a cell death process that displays features of necroptosis and provides an ICD stimulus to the adaptive immune system compartment (Figure 1).

Mechanistically speaking, since melanomas often retain wild-type p53, we reasoned that this powerful tumor suppressor could be recruited to assist in the treatment and also promote high levels of transgene expression from our adenoviral vector, which employs a synthetic p53-responsive promoter called PGTxβ. In fact, we have shown that the p53-responsive promoter outperforms the typically employed cytomegalovirus (CMV) immediate-early promoter or retroviral long terminal repeat (LTR), providing 5 – 7 times higher transgene expression.[5] In our current work, an additional improvement was made to this nonreplicating, serotype 5 adenoviral vector platform, the use of a modified adenoviral fiber protein containing the RGD tripeptide. With this alteration, the adenoviral vector no longer depends on the CAR for entry and instead interacts with integrins. Thus, the viral vector developed for this study offers robust transgene expression as well as ample tropism.

Cooperation between the p53/Arf and IFN pathways has been reported previously, showing that type I IFN's antiviral defense is enhanced by p53 activity, and that type I IFNs can activate p53 at the transcriptional and post-translational levels.[6–8] We expect that p19Arf+IFNβ should cooperate to activate p53, promote expression from the viral vector due to the p53-responsive promoter, bring about cell death and activate the immune system.

Leading up to this study, we were the first to show that combined, but not individual, p19Arf+IFNβ gene transfer enhanced killing of B16 mouse melanoma cells *in vitro* and *in vivo*.[9] We had also shown several aspects of an antitumor immune response, mediated by natural killer cells, CD4+ and CD8+ T lymphocytes, that occurred only when combined gene transfer was applied in prophylactic and therapeutic vaccine models or in *in situ* gene therapy of primary tumors.[10,11] However, we did not have an in-depth understanding of the tumor cell's molecular response to gene transfer nor had the cell death mechanism been thoroughly explored.

Among our recent findings,[4] we show that p19Arf supplied by adenovirus-mediated gene transfer sensitized B16 cells to the effects of IFNβ secreted by neighboring cells. That is, IFNβ's bystander effect was enhanced when p19Arf was present. We next explored the importance of gene transfer as compared to the use of drugs for the stimulation of the p53/Arf and IFN pathways. While Nutlin-3 (which, similar to p19Arf, frees p53 from MDM2) could substitute p19Arf gene transfer,

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News and Commentary

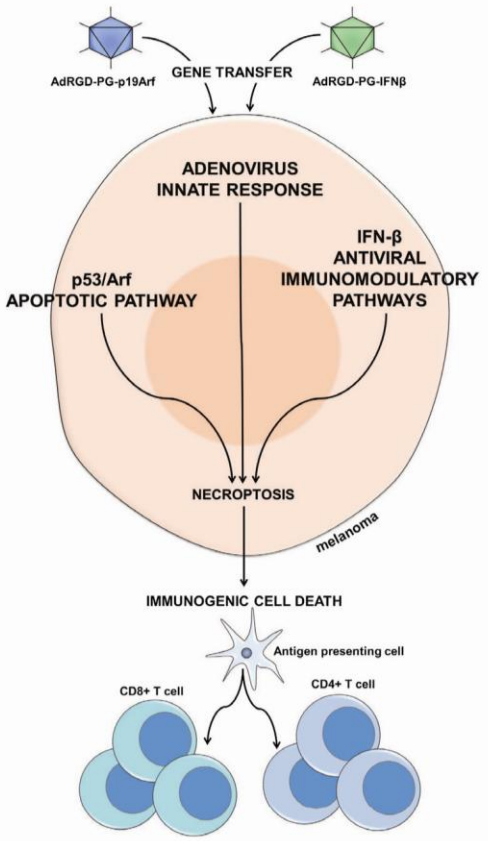

Figure 1 Proposed model for the mechanisms culminating in cell death and immune activation upon p19Arf and interferon-β gene transfer. Initially, on remediation of p19Arf by the AdRGD-PGp19Arf adenoviral vector, p53 becomes free-form MDM2 and activates its pro-apoptotic pathway, evidenced by upregulation of its target genes, caspase-3 activity and Bax protein levels. Just as Arf is not strong enough to cause massive cell death on its own, IFNβ by itself mainly inhibits proliferation and potentiates an antiviral and immunostimulatory response, facilitated by the presence of adenovirus components. Combined activation of these pathways provides a stimulus strong enough for the efficient killing of melanoma cells. This process of cell death displays features of necroptosis, suggested by RIP3 expression, upregulation of the TNF receptor, absence of caspase-3 activity and most importantly, immunogenic cell death markers (ATP, calreticulin, HMGB1), along with IFNβ, to promote an antitumor immune response mediated by NK cells, CD4+ and CD8+ T lymphocytes

and Poly (I:C) or recombinant IFNβ protein could replace the vector encoding IFNβ, the presence of the adenoviral vectors was necessary for the induction of high levels of cell death. Interestingly, Poly (I:C)+Nutlin-3 induced significant cell killing only in the presence of an innocuous adenoviral vector encoding eGFP, suggesting involvement of an antiviral response. Indeed, we confirmed the induction of genes associated with antiviral response, including Dram1, Chop, Nlrc5 and ISG15, especially so in the presence of combined p19Arf+IFNβ gene transfer, both *in vitro* and *in vivo*.

While markers of apoptotic cell death (AnnexinV staining, upregulation of Bax and caspase 3 activity), were significantly enhanced when cells were treated with just p19Arf, addition of IFNβ to p19Arf treatment altered the cell death as compared to the transfer of p19Arf alone. Strikingly, inhibition of pan-caspase activity with Z-VAD-FMK drastically increased cell killing upon p19Arf of IFNβ single-gene transfer and had no effect on the p19Arf+IFNβ combination, thus suggesting that alternate routes of cell death were involved. In fact, RIP3, a key mediator of necroptosis, and Tnfrsf1A, an activator of the necrosome complex, were specifically induced upon combined p19Arf and IFNβ gene transfer, indicating necroptosis as a possible mechanism of cell death. Also, the induction of all three classic ICD markers (calreticulin exposure, ATP secretion and HMGB1 release) was seen only upon combined gene transfer, in agreement with recent findings showing that necroptotic cells undergo ICD upon chemotherapy treatment.[12]

Moreover, as revealed by microarray analysis, cooperation between the p53/Arf and IFNβ pathways in the context of adenoviral transduction resulted in the induction of an antiviral response. Remarkably, only p19Arf+IFNβ treatment induced gene expression signatures related to the p53 signaling pathway and apoptosis as well as immune response, response to virus and antigen processing. This may explain why high levels of cell death in addition to release of immunogenic markers were only seen by this combined treatment. Also, all of these treatments were able to inhibit expression of genes related to cell cycle function.

The data described here provide a molecular framework that supports the successful immunotherapy described in our previous studies where vaccines or *in situ* gene therapy with p19Arf+IFNβ could reduce the progression of challenge tumors.[10,11] We propose that our approach may provide advantages not seen with existing immunotherapies. For example, gene transfer is not associated with strong adverse reactions, such as the cardiotoxicity seen with a bona fide ICD inducer like doxorubicin. Since we do not employ an oncolytic vector, impedance of virus spread due to the immune response is not a concern.

Even with the advances detailed in our study, additional points remain unexplored. Some authors suggest that prolonged binding to Tnfrsf1A may result in exacerbated TAK1 activation, which in turn results in RIP3 phosphorylation and activation.[13] We do not discard the possibility that DAI/ZBP1, the DNA-dependent activator of interferon regulatory factors, may be involved in the RIP1-independent induction of RIP3, since the presence of adenovirus, a possible source of cytoplasmic dsDNA, was important for the induction of high levels of cell death. However, the involvement of TAK1 and DAI in our immunotherapy remains to be determined experimentally.

Certainly, further development is necessary before we can suggest the application of our approach in the clinical setting. Even so, the work described here was critical for exposing the advantages that combined p19Arf and IFNβ gene transfer brings to the cancer immunotherapy arena.

Conflict of Interest

The authors declare no conflict of interest.

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POINT OF VIEW

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Uncovering the immunotherapeutic cycle initiated by p19Arf and interferon-β gene transfer to cancer cells: An inducer of immunogenic cell death

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ABSTRACT
Simultaneous reestablishment of p53/p19^{Arf} and interferon-β pathways in melanoma cells culminates in a cell death process that displays features of necroptosis along with the release of immunogenic cell death molecules and unleashes an antitumor immune response mediated by natural killer cells, neutrophils as well as CD4$^+$ and CD8$^+$ T lymphocytes.

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Adenovirus; gene therapy; immunogenic cell death; immunotherapy; melanoma; p53

Introduction

During the multistep process of tumorigenesis, cancer cells must acquire several new characteristics, including the capability to resist intrinsic cell death mechanisms and to escape from immune surveillance in order to establish a highly immune suppressive microenvironment.[1] Such characteristics are well documented in human melanoma, that retains wild-type p53 in 90% of cases, although inactivated due to elevated levels of MDM2, seen in 56% of cases,[2] or loss of p14^{ARF}, which occurs in approximately 50% of cases.[3] Interestingly, the Arf (*CDKN2A*) and interferon-β (IFN-β) genes are both located on human chromosome 9p21 and their frequent loss points to their importance in preventing tumorigenesis. For melanoma, hemizygotic deletion of 9p21 was observed in 12 of 14 cases,[4] which may reflect loss of *CDKN2A* or IFN-β alone or in combination.[5] Thus, the remediation of Arf and IFN-β would not only reinstate the expression of some of these genes, but complement the activity of endogenous p53 and promote an antitumor immune response.

Following this line of thought, we have explored the p53/Arf and IFN-β pathways, developing a set of unique adenoviral vectors that use a p53 responsive promoter and encode the p19^{Arf} tumor suppressor protein (p53 functional partner) and IFN-β (immunomodulatory cytokine).[6] As discussed here, the combined gene transfer of these factors to tumors harboring wild-type p53 creates an interplay between (i) transgene control, (ii) p53/Arf apototic stimulus and (iii) an IFN-β antiviral inflammatory context, that together unleashes immunogenic cell death (ICD) as well as a systemic innate and adaptive immune attack against cancer cells at primary and distant sites.

Evidence for cooperation between the p53/Arf and interferon-β pathways

The canonical antitumor mechanism of p14^{ARF} (p19^{Arf} in the mouse) involves its induction by several forms of oncogenic stress and its association with MDM2, thus inhibiting the degradation of p53.[7] In this way, ARF enables p53 activity, such as triggering growth arrest or apoptosis. In addition, ARF has p53-independent functions, such as induction of cell cycle arrest, senescence, apoptosis and regulation of autophagy.[8]

IFN-β, as a member of the type I IFN family, is a cytokine initially known for its antiviral effects, but also exerts an important role in antitumor immune responses.[9] Upon binding to IFNAR1/2 transmembrane receptors, IFN-β promotes the transcription of hundreds of IFN-stimulated genes (ISGs), for example, Interleukin-1β (IL-1β), IFN-γ, and pro-apoptotic proteins, like FAS and TRAIL.[10] It can also induce the expression of major histocompatibility complex (MHC) class I molecules, maturation of dendritic cells and increase cytotoxic activity of natural killer (NK) cells and CD8$^+$ T lymphocytes.[11]

Moreover, it has been observed that the p53/Arf and type I IFN pathways intersect and even cooperate. For example, the presence of p53 has been shown to boost the antiviral response when cells were exposed to recombinant type I IFN.[12,13] The *TP53* promoter contains an IFN-stimulated response elements (ISRE) and p53 transcription is activated by IFN-α/β, while p53 protein is stabilized upon cellular exposure to type I IFN.[12] The transcription of IRF-5 and IRF-9, which are ISGs, are also known to be activated by p53, contributing to anti-viral defense.[14,15] However, it has also been suggested that cell death in response to IFN-α/β is actually dependent on ARF, not p53.[16] While these examples show interplay between the p53/

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Arf and type I IFN pathways, cooperation between them has not been exploited as a therapeutic target in cancer.

Harnessing adenoviral mediated gene transfer of p19^{Arf} and interferon-β

To successfully mediate the transfer of the Arf and IFN-β cDNAs into tumor cells, our group developed an adenoviral vector where transgene expression is controlled by a p53-responsive promoter, called PGTx-β (or simply PG) , which expresses the transgene 5–7 times more than a traditional constitutive promoter derived from cytomegalovirus.[17] Interestingly, when PG promotes the expression of p53, an autoregulatory, positive feedback mechanism is established, providing exceptionally high levels of p53 expression and superior tumor cell killing.[18] This positive feedback also occurs when PG promotes the expression of p19^{Arf}. In this scenario, p19^{Arf} was shown to stabilize endogenous p53 in B16 (mouse melanoma) and C6 (rat glioma) cells, both of which harbor endogenous wild-type p53. Interestingly, transfer of p53 to B16 cells did not show an antitumor response, in contrast to p19^{Arf} that significantly affected cell viability.[19]

As demonstrated in a later study, the response to p19^{Arf} was enhanced when combined with IFN-β. In fact, only combined gene transfer, but not the individual treatment, with p19^{Arf} and IFN-β (p19^{Arf}+IFN-β) resulted in massive cell death of B16 cells *in vitro*, as well as in a model of *in situ* gene therapy, where adenoviral vectors were injected directly into subcutaneous (s.c) tumors and significantly inhibited tumor progression and prolonged survival.[6] Although this data looked promising, a key question regarding the combination of p19^{Arf} with IFN-β was left unanswered: since IFN-β is already known to promote immune activation, could the addition of p19^{Arf} provide an immunotherapeutic benefit? The hypothesis was that, if the p19^{Arf}+IFN-β gene transfer could provide additional immunomodulatory stimulus, it would come from danger associated molecules released through the death process.

In light of this question, we explored a prophylactic cancer vaccine model in which B16 cells were transduced *ex vivo* with the p19^{Arf}+IFN-β combination and, while at the beginning of the cell death process, inoculated (s.c) in the left flank (vaccine site) of immune competent syngeneic C57BL/6 mice. Seven days after the last vaccine, fresh untreated B16 cells were inoculated in the contralateral flank (tumor challenge site).[20] As expected, mice immunized with p19^{Arf}+IFN-β treated cells showed a dramatic decrease in challenge tumor progression, when compared with control groups. Furthermore, we found that this antitumor protection was maintained even when the challenge was performed 73 days after vaccination, indicating the generation of memory response. Also, tumors displayed a high infiltration of hematopoietic CD45$^+$ cells, and were being rejected in a CD4$^+$ and CD8$^+$ T-cell-dependent manner, thus proving the immunological nature of this response.[20]

Unexpectedly, in the vaccine site, we observed that tumors grew out in a much delayed fashion when mice received three vaccine inoculations with p19^{Arf}+IFN-β treated cells, suggesting that B16 clones with tumorigenic potential remained after treatment and were being kept under immune control, until eventually escaping to later establish the tumor. Seeking to understand this, cells treated with p19^{Arf} and IFN-β alone or combined were inoculated in C57BL/6 (immune competent), nude (athymic mice that lack mature T lymphocytes) or NOD-SCID (lacking both T and NK cells) mice, without further tumor challenge. Surprisingly, tumor formation was completely abrogated only in hosts with functional NK cells that were inoculated with p19^{Arf}+IFN-β treated cells. In accordance with this, immunosuppression with dexamethasone, an inhibitor of NK cell cytotoxic functions,[21] recapitulated this result and RT-qPCR analysis of *in vitro* treated B16 cells revealed upregulation of IL-15, ULBP1 NK cell activating receptor, KILLER/DR5 and FAS/APO-1 death receptors, therefore, supporting the notion that these cytotoxic innate immune cells were being activated by the p19^{Arf}+IFN-β treated B16 cells and mediated immune rejection at the vaccine site.[20]

Superior immune stimulus of the combined gene therapy, in comparison with IFN-β treatment, could not be detected when evaluating the magnitude of the T cell response, as popliteal lymphocytes secreted similar levels of Th1 related cytokines (IFNγ, IL-2 and tumor necrosis factor-α), indicating no evident advantage for the addition of p19^{Arf}. Interestingly, when a therapeutic vaccine was used, (i.e. vaccination was performed after tumor challenge), we observed a greater reduction in tumor progression in the p19^{Arf}+IFN-β group, when compared with the treatment with IFN-β alone.[20]

Additional evidence for the benefit of the combined treatment was obtained from a second immunization model, in which adenoviral vectors were directly injected into established s.c. LLC1 tumors (lewis lung carcinoma, wt p53) and, after four rounds of treatment, a contralateral tumor challenge was performed with naïve LLC1 tumor cells. The hypothesis was that immunization in an established primary tumor, where immunosuppression is at its prime, would be more challenging for an effector immune response to be orchestrated and thus, could reveal if the p19^{Arf}+IFN-β actually provides an immune advantage over IFN-β single treatment. Indeed, even though primary tumors from both groups presented similar growth impairment, tumors from the p19^{Arf}+IFN-β group showed remarkably reduced progression in the secondary site.[22] Here, just as seen in the therapeutic vaccine model, the combination provided superior immunological protection.

Seeking to better understand the mechanisms behind immune stimulation triggered by the p19^{Arf}+IFN-β combination, we performed microarray analysis of *in situ* treated LLC1 tumors. Differentially expressed genes revealed a strong immune response and chemotaxic signature with the involvement of neutrophils and CD8$^+$ T cells, as evidenced by the upregulation of CCL3, CXCL3, IL-1α and IL-1β. Also, immunohistochemical quantification of CD11b$^+$ Ly6G$^+$ neutrophils showed an increase exclusively in the p19^{Arf}+IFN-β group. Moreover, antibody-mediated granulocyte depletion throughout the treatment regime could greatly impair the inhibition of tumor growth caused by p19^{Arf}+IFN-β gene transfer, demonstrating the critical role that this population plays in our model.[22] Importantly, this result does not exclude the involvement of CD8$^+$ T lymphocytes or NK cells.

Next, we evaluated the molecular mechanisms of death induced by p19^{Arf} and IFN-β alone or in combination. One key observation was that cell death in response to p19^{Arf}+IFN-β is

only possible in the context of adenoviral gene transfer, since the substitution of both $p19^{Arf}$ and IFN-β gene transfer for pharmacological approaches, like Nutlin-3 (a compound that binds to Mdm2 and releases p53), recombinant IFN-β (IFN-R) or Poly (I:C) (a toll-like receptor 3 agonist and inducer of endogenous IFN-β expression), did not cause high levels of cell death. Interestingly, when only one gene was substituted by a pharmacological mimetic, but the other was still transferred by the adenoviral vector, high levels of cell death were detected, confirming the importance of the adenovirus-mediated gene transfer in cell death induction. Indeed, the expression of genes related to an antiviral response (*Nlrc5*, *Dram1*, *Isg15* and *Chop*) was detected only after cells were treated with $p19^{Arf}$ and IFN-β together.[23]

We next analyzed apoptosis markers and detected exposure of Annexin-V and Bax expression mainly after $p19^{Arf}$ treatment. The addition of IFN-β seemed to modify the cell death pathway induced by $p19^{Arf}$, since increased expression of Rip3 and *Tnfrsf1a* in cells treated with the $p19^{Arf}$+IFN-β combination was observed. In fact, the treatment of B16 cells with Z-VAD-FMK, a pan-caspase inhibitor, before gene therapy did not inhibit cell death, but increased its levels, indicating again an alternative mechanism of cell death. The increase in Rip3 and *Tnfrsf1* expression points to the occurrence of necroptosis, which was recently associated with the activation of ICD and is proposed to be a molecular mechanism of cell death that provides both antigenicity and adjuvancy stimuli to dendritic cells.[24] In fact, detection of all the three *bona fide* markers of ICD (exposure of Calreticulin, secretion of ATP and release of HMGB1) were only observed after $p19^{Arf}$+IFN-β combined treatment, possibly explaining the immunomodulatory superiority of the combination.[23]

In support to these findings, we evaluated the gene signature induced by each treatment. Global gene expression analysis revealed that all treatments ($p19^{Arf}$, IFN-β or the combination of both) inhibited genes related to cell cycle progression, while only $p19^{Arf}$+IFN-β induced expression of genes related to the p53 signaling pathway and apoptosis, as well as immune response, response to virus and antigen processing.[23]

The immunity cycle initiated by combined gene transfer of $p19^{Arf}$ and interferon-β to cancer cells

Based on the data presented above, we propose the following hypothesis (Fig. 1): *first*, at the immunization site either by injection of *ex vivo* treated cells or *in situ* gene therapy, the reactivation of the p53/Arf pro-apoptotic pathway in the presence of an antiviral and immunogenic context, that were elicited by the adenoviral vector and IFN-β signaling, starts the cell death process along with the upregulation of IL-15, ULBP1, FAS/APO1, and KILLER/DR5 genes, consequently providing positive stimulus to NK cells to recognize and kill treated tumor cells. *Second*, the cell death process itself and the upregulation of CCL3, CXCL3 and IL-1β in the tumor mass participates in the recruitment of $CD11b^{+}$ $Ly6G^{+}$ neutrophils that may function in the reshaping the tissue after injury, favor the infiltration of $CD8^{+}$ T cells, directly kill tumor cells or even inhibit angiogenesis.[25] The exact function of neutrophils in our model

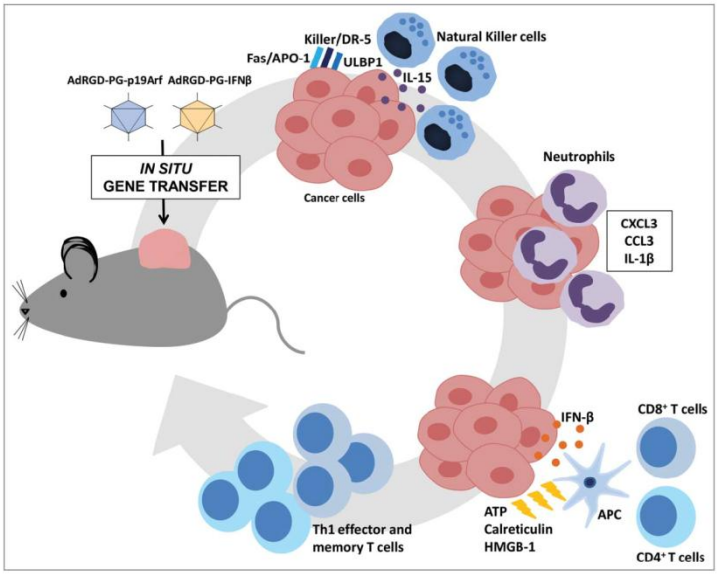

Figure 1. Proposed mechanism for the immunotherapeutic cycle induced upon combined gene transfer of $p19^{Arf}$ and interferon-β. *In situ* injection of both AdRGDPG-$p19^{Arf}$ and AdRGDPG-IFN-β adenoviral vectors into the tumor mass results in the reestablishment of p53/Arf pro-apoptotic and interferon-β pathways and culminates in a cell death process that: (i) upregulates Interleukin-15, ULBP1 and KILLER/DR5 to stimulate natural killer cells, (ii) recruits $CD11b^{+}$ $Ly6G^{+}$ neutrophils by the up regulation of CCL3, CXCL3 and Interleukin-1β, (iii) and together with the release of calreticulin, ATP and HMGB1 induces a Th1 cytotoxic immune response and establishment of immunological memory.

remains to be elucidated. *Third*, secretion of high levels of IFN-β together with the release of calreticulin, ATP and HMGB1, should provoke profound modulatory effects on tumor associated APCs to achieve an optimal maturation state and, upon encounter with naïve T lymphocytes, induce a Th1 cytotoxic immune response, which was evidenced by the secretion of IFNγ and TNF-α. We speculate that the main importance of these primed tumor-specific $CD4^+$ and $CD8^+$ T cells is not at the primary tumor site, but at the distant challenge tumor site, that mimics metastatic foci, since an increase of adaptive immune negative regulators, such as PD-L1, have been found to be induced at the primary site due to the IFN-β inflammatory response.[22,26] *Finally*, establishment of immunological memory has the potential to attack malignant cells that could eventually relapse after therapy, providing long lasting immune protection.

So far, we believe that we have gathered enough functional evidence to characterize the adenovirus mediated gene transfer of $p19^{Arf}$ together with IFN-β as a novel cancer immunotherapy strategy. To the best of our knowledge, no other gene transfer strategy using a replication deficient viral vector has been shown to induce ICD, a molecular mechanism already reported with chemotherapy (e.g., doxorubicin), radiotherapy and even with oncolytic replication-competent vectors, such as the Newcastle disease virus.[27,28]

We also have successfully identified critical players of our therapy: NK cells, $CD4^+$ and $CD8^+$ T lymphocytes, neutrophils and ICD molecules. However, the kinetics, location and precise mechanisms by which all these components cooperate needs to be understood in greater detail. Indeed, it has been reported that, in response to IL-15, NK cells can assume a helper phenotype and influence T cell priming toward a Th1 response.[29] Neutrophils can also modulate T cell immunity on several levels, including recruitment, activation and effector function.[25] Most importantly, since the $p19^{Arf}$+IFN-β combination provides both ICD and IFN-β stimulus, it will be interesting to investigate how the DC compartment is being affected. As recently demonstrated, DC vaccines based on ICD provoke a T cell-driven rejection of high-grade gliomas[30] and may represent a therapeutic opportunity that can be further explored. Nevertheless, as type I IFNs have been shown to regulate PD-L1 expression on macrophages,[26] association of $p19^{Arf}$+IFN-β with PD-1/PD-L1 checkpoint blockade immunotherapy is expected to provide great therapeutic benefit. Even though much remains to be investigated, potential therapeutic applications of our $p19^{Arf}$+IFN-β approach deserve further development.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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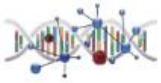

Fundamentos de Oncologia Molecular tem a base de seu texto no momento em que Disciplina de Oncologia da Faculdade de Medicina da Universidade de São Paulo foi transferida para o Instituto do Câncer do Estado de São Paulo. Ou seja, o ensino de Oncologia, da graduação, passando pela Residência Médica à pós-graduação, concentrou-se em um só local para onde também chegaram os laboratórios de pesquisa, estes vinculados às atividades da pós-graduação.

Essa reformulação aproximou os pós-graduandos de residentes e alunos de graduação. Catalisaram-se interações. Foi criado um ambiente favorável para o exercício e a discussão da didática nos currículos de oncologia para estudantes das Ciências da Saúde.

Com isso, obteve-se uma proposta curricular em Oncologia Molecular inspirada nos currículos internacionais. Os temas discutidos e a sua abordagem nada mais são que os capítulos deste livro. Verdadeira fonte de referência de conceitos fundamentais em oncologia e com a discussão das perspectivas de evolução.

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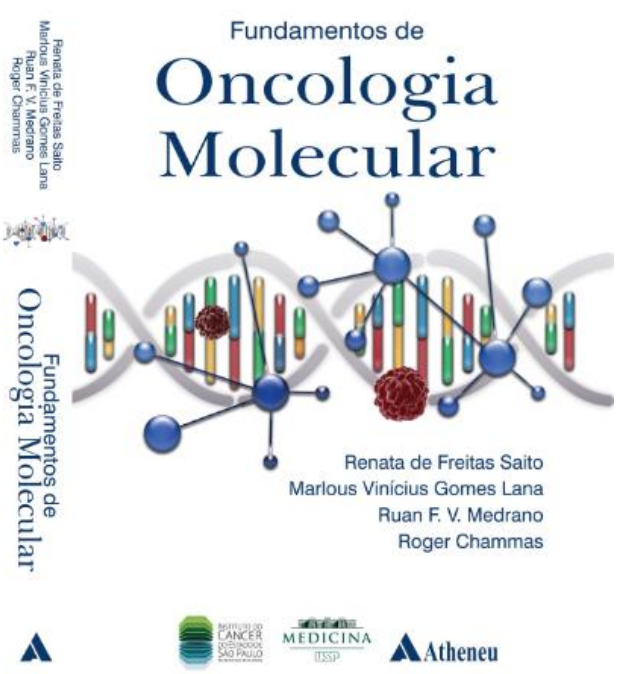

Imunologia de Tumores 14

Ruan F. V. Medrano
Elaine Guadelupe Rodrigues

INTRODUÇÃO

No decorrer deste livro aprendeu-se que os mamíferos podem impedir o desenvolvimento de células tumorais através de mecanismos como reparo de DNA e apoptose (morte celular). Estes e outros processos atuam de maneira efetiva para impedir que alterações ou mutações que possam favorecer a tumorigênese se perpetuem nas células-filhas. E são desencadeados pela própria célula que sofreu o insulto oncogênico para atuar contra si mesma cometendo, se não for possível o reparo, um ato de suicídio, sendo por isso caracterizados como mecanismos intrínsecos (passam-se no interior das células). Porém, caso essas defesas intrínsecas falhem, o organismo ainda possui uma barreira extrínseca: o sistema imunológico.

O sistema imunológico é conhecido por detectar e gerar respostas eficazes na eliminação de agentes invasores, ou seja, organismos estranhos ao hospedeiro, principalmente, os que representam perigo, como bactérias, fungos e vírus. De fato, a primeira demonstração de que o sistema imune é capaz de combater tumores utilizou-se do mesmo conceito de agente invasor. Em 1891, o médico William Coley tratou com sucesso alguns pacientes com sarcomas, injetando diretamente nos tumores produtos bacterianos de *Streptococcus pyogenes e Serratia marcescens*, transformando assim os tumores em algo estranho e perigoso ao hospedeiro, que deveria ser eliminado.

Este breve relato levanta importantes questões, como: do ponto vista imunológico, seriam os tumores semelhantes aos agentes invasores? Como ocorreu a resposta imune na terapia de Coley? Como os tumores reagiram a esta resposta? Dessa forma, ao final deste capítulo espera-se responder estas perguntas para que o leitor compreenda que: (i) os tumores, apesar de serem algo próprio do hospedeiro, são vistos como perigo; (ii) a resposta imune antitumoral envolve tanto componentes imunes inatos quanto adaptativos; (iii) porém, o tumor subverte estes mecanismos imunes e (iv) portanto, a relação existente entre o tumor e o sistema imune pode ser considerada como um processo dinâmico que está em constante evolução.

DESTAQUES

- A relação entre o sistema imunológico e o câncer pode ser ilustrada pela teoria da imunoedição tumoral.
- Células *natural killers*, linfócitos T-*helper* e T citotóxicos são as principais células efetoras de resposta imune antitumoral.
- A evasão imune realizada pelos tumores envolve linfócitos T reguladores, células mieloides supressoras e as moléculas IDO e interleucina 10.

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A CEUA da Faculdade de Medicina da Universidade de São Paulo, em sessão de 11/12/2014, **APROVOU** o Protocolo de Pesquisa nº **165/14** intitulado: **"Associação da imunoterapia mediada pela transferência gênica de p19Arf e Interferon-beta com morte imunogênica induzida pelo quimioterápico Doxorrubicina para o tratamento do câncer"** que utilizará **300** animais da espécie **camundongo C57Bl/6** e **60** animais da espécie **camundongo Balb/c Nude**, apresentado pelo Laboratório de Oncologia Experimental - ICESP

Cabe ao pesquisador elaborar e apresentar à CEUA-FMUSP, o relatório final sobre a pesquisa, (Lei Procedimentos para o Uso Científico de Animais - Lei Nº 11.794 -8 de outubro de 2008).

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Pesquisador (a) Executante: Ruan Felipe Vieira Medrano

CEUA-FMUSP, 11 de Dezembro de 2014

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Personal Statement

Ruan Felipe Vieira Medrano was born in November 18th 1985, in Brazil. In 2006, he started his under-graduate studies in Biomedicine at *Uniararas*, where under the supervision of Prof. Camila Andrea de Oliveira he also initiated his scientific career by working with the identification of single nucleotide polymorphisms (SNPs) associated with nonsyndromic hearing loss. In 2010, after obtaining his bachelor degree, he joined the lab of Prof. Bryan Strauss to start his Master`s in Oncology at *Universidade de São Paulo*. It was during this time, investigating the gene transfer of p19Arf/Interferon-β as cancer immunotherapy strategy, that he developed his fascination with tumor immunology and decided to pursue this field in order to become an established researcher. In 2013, he continued working in the Dr. Strauss lab as well as his education in Oncology at *Universidade de São Paulo* and started his Ph.D. There he explored potential benefits of associating p19Arf/IFNβ immunotherapy with immunogenic cell death inducers or with checkpoint blockade. And in 2016, he made an internship with Prof. Robert Schreiber at Washington University in St. Louis, USA. Where he worked with optimization and development of neoantigen based cancer vaccines. As for his plans for the near future, he intends to finish his Ph.D and to start a postdoc in order to deepen his studies in tumor immunology.

Education

Bachelor in Biomedicine (2006-2009)

- Centro Universitário Herminio Ometto – Uniararas

Master in Oncology (2010-2013)

- Universidade de São Paulo – USP

Ph.D in Oncology (2013-on going)

- Universidade de São Paulo - USP

Research Internship

In the Dr. Robert D. Schreiber laboratory, Pathology and Immunology Department, Washington University in Saint Louis, USA. (2016)

Professional Membership

Member, American Association of Cell and Gene Therapy - ASGCT (2013)
Member, American Association for Cancer Research – AACR (2016)
Member, European Academy of Tumor Immunology- EATI (2016 – on going)
Member, Brazilian Society of Immunology – SBI (2017 – on going)

Honors and Awards

Honorable Mention, for the abstract "Avaliação in vitro da atividade antioxidante de componentes da dieta", 2º Congresso Científico Uniararas - 1º Congresso de Iniciação Científica PIBIC - CNPQ, Uniararas. (2007)

Honorable Mention, for the abstract "Induction of cell death by p19Arf and IFN-beta in tumor cells resistant to p53 gene therapy", 4° Simpósio de Imunobiologia de Tumores da Universidade Estadual Paulista – UNESP. (2011)

Honorable Mention, for the abstract "Associating cell death and immune stimulation with the p19Arf and Interferon-beta combination**".** Department of Oncology, USP. (2013)

Awarded Abstract. "Treatment with the combination of $p19^{Arf}$ and interferon-beta elicits an anti-tumor immune response", 1st ASGCT Symposium. (2013)

Distinguished Student, for the paper "Combined p19Arf and interferon-beta gene transfer enhances cell death of B16 melanoma *in vitro* and *in vivo*", Department of Oncology, USP. (2014)

Distinguished Student, for the authorship of the "Tumor immunology" chapter in the **"**Principles in Molecular Oncology" book, Department of Oncology, USP. (2016)

V Maria Mitzi Brentani Award, for the abstract "Potentiation of p19Arf/Interferon-β immunotherapy through its association with doxorubicin: combining two immunogenic cell death inducers for the treatment of cancer", Department of Oncology, USP. (2017)

Peer-Reviewed Publications

1. MERKEL, C. A. ; MEDRANO, R. F. V. ; BARAUNA, V. G. ; STRAUSS, B. E. Combined p19Arf and interferon-beta gene transfer enhances cell death of B16 melanoma in vitro and in vivo. **Cancer Gene Therapy,** p. 21-28, 2013.

2. BAJGELMAN, M. C., MEDRANO, R. F. V., CARVALHO, A. C. V., STRAUSS, B. E. AAVPG: A vigilant vector where transgene expression is induced by p53. **Virology**, v. 447, p. 166-171, 2013.

3. MEDRANO, R.F.V., OLIVEIRA, C.A. A Practical guide for the tetra-primer ARMS-PCR technique development**. Molecular Biotechnology**. 2014.

4. GRILLO, A.P., CARVALHO, G.Q., SILVA-COSTA, S.M, MEDRANO, R.F.V., OLIVEIRA, C.A Novel Single Nucleotide Polymorphisms of the *GJB2* and *GJB6* Genes Associated with Nonsyndrmic Hearing Loss**. Biomed Research Internacional**. 2015.

5. MEDRANO, R.F.V. CATANI, J.P.P., RIBEIRO, A.H. STRAUSS, B. E. E. Vaccination using melanoma cells treated with p19arf and interferon beta gene transfer in a mouse model: a novel combination for cancer immunotherapy. **Cancer Immunology and Immunotherapy,** v. 65, p. 371-382, 2016.

6. CATANI, J.P.P; MEDRANO, R.F.V. ; HUNGER, A; DEL VALLE, P; ADJEMIAN, S; ZANATTA, D,B; KROEMER, G ; COSTANZI-STRAUSS, E; STRAUSS, B.E. . Intratumoral Immunization by p19Arf and Interferon-β Gene Transfer in a Heterotopic Mouse Model of Lung Carcinoma. **Translational Oncology**, v. 9, p. 565-574, 2016.

7. RIBEIRO, A.H; MEDRANO, R,F.V; ZANATA, D.B; DEL VALLE, P; SALLES, T; FERRARI, D. CONSTANZI-STRAUSS, E. STRAUSS, B.E. Reestablishment of p53/Arf and Interferon-β pathways mediated by a novel adenoviral vector potentiates antiviral response and immunogenic cell death. **Cell Death Discovery**, Mar 20;3:17017, 2017.

8. RIBEIRO, A.H; MEDRANO, R,F.V; STRAUSS B.E. Harnessing combined p19Arf and interferon-beta gene transfer as an inducer of immunogenic cell death and mediator of cancer immunotherapy. **Cell Death Disease**. May 11;8(5):e2784, 2017.

9. MEDRANO, R,F.V; RIBEIRO, A.H; CATANI, J.P.P; STRAUSS B.E. Uncovering the immunotherapeutic cycle initiated by p19Arf and interferon-β gene transfer to cancer cells: An inducer of immunogenic cell death. **Oncoimmunology**. May 19;6(7):e1329072, 2017.

10. MEDRANO, R,F.V*; RIBEIRO, A.H*; MENDONÇA, S.A; BARBUTO, J.A.M; STRAUSS, B.E. Immunomodulatory and antitumor effects of type I interferons and their application in cancer therapy. **Oncotarget**. Jul 25;8(41):71249-71284, 2017. * contributed equally

Book

SAITO, R., LANA, M.G., MEDRANO, R.F.V., CHAMMAS, R. **Fundamentos de Oncologia Molecular** (Principles in Molecular Oncology). Atheneu. 2015. ISBN 978-85-388-0684-4

Book Chapter

MEDRANO, R.F.V and GUADELUPE, E.R. **Imunologia de Tumores** (Tumor Immunology). Fundamentos de Oncologia Molecular. Atheneu. 2015.

Proffered Lectures, Classes and Courses

- **Course,** Gene transfer techniques and gene reporter assays**,** 2011.
- **Class**, Cell death mechanisms, 2011.
- **Course**, Cell death pathways and investigation methods, 2012.
- **Lecture**, Pursuing a scientific career: what I have learned so far, 2012.
- **Class**, Cell cycle in cancer, 2013.
- **Lecture**, Harnessing the immune system for the treatment of cancer, 2013.
- **Lecture**, Gene transfer of p19Arf and interferon-beta as cancer immunotherapy, 2014.
- **Class**, Tumor immunology, 2015.
- **Course**, Mice models for cancer research, 2015.
- **Lecture**, Immune context in human cancers, 2015.
- **Class**, Cancer immunotherapy, 2017.
- **Lecture.** Setting the framework for personalized cancer immunotherapy: the role of neoantigens in tumor immunity, 2017.
- **Lecture,** Harnessing combined p19Arf and interferon-beta gene transfer as an inducer of immunogenic cell death and mediator of cancer immunotherapy, 2017.

- **Lecture**, Remediation of the p53/Arf and interferon-β pathways as a novel cancer immunotherapy strategy: a gene transfer approach, 2017.

Organization of Scientific Events

- **Workshop**, Planning committee, 3º Workshop in Biotechnology. Uniararas, 2007.
- **Conference**, Planning committee, 1st Biomedicine Academic Week. Uniararas, 2008.
- **Course**, Student coordinator, I Course on Molecular Oncology, USP, 2011.
- **Conference**, Planning committee. IV Oncology Program Meeting, USP. 2012.
- **Course**, Student coordinator, III Course on Molecular Oncology, USP, 2015.

Academic Activities

- **Class assistant**, Biochemistry and Genetics, Uniararas, 2007.
- **Student coordinator** of the "Advanced study group for molecular and cellular biology", Uniararas, 2007.
- **Vice-president** and **scientific organizer** of the Biomedicine Academic League, Uniararas, 2008-2009.
- **Student representative**, Department of Oncology, USP, 2012.

Fellowships

- Undergrad scientific iniciation fellowship from Programa Institucional de Bolsas de Iniciação Científica - PIBIC- CNPq, 2008-2009
- Master degree fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo- FAPESP, 2010- 2013
- Ph.D fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo- FAPESP, 2013- on going
- Internship fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo- FAPESP, 2015-2016

Participation in Scientific Events

1. **Poster presentation** at 42h Congress of the Brazilian Society of Immunology (Mucosal Immuno). Brazil, 2017.
2. **Poster presentation** at Third CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference: Translating Science into Survival. Germany, 2017.
3. **Poster presentation** at I Workshop Autophagy and Diseases, Brazil, 2017.
4. **Poster presentation** at AACR International Conference on Translational Cancer Medicine, held in cooperation with the Latin American Cooperative Oncology Group (LACOG). Brazil, 2017.
5. **Attendance** at Immunology Program Retreat from Washington University School of Medicine. USA, 2016.
6. **Attendance** at Second CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference: Translating Science into Survival. USA, 2016.
7. **Poster presentation** at VII Jornada do Programa de Pós-Graduação em Oncologia. 2016.
8. **Poster presentation** at Keystone Symposia meeting on Tumor Immunology: Multidisciplinary Science Driving Combination Therapy. Canada, 2015.
9. **Poster presentation** at VI Jornada do Programa de Pós-Graduação em Oncologia.

Brazil, 2014.

10. **Short talk** at Challenges and solutions in cancer Conference, Brazil. 2014
11. **Short talk** at V Jornada do Programa de Pós-Graduação em Oncologia. Brazil, 2013.
12. **Poster presentation** at São Paulo School of Advanced Sciences: Advances in Molecular Oncology. Brazil, 2013.
13. **Poster presentation** at IV Jornada do Programa de Pós-Graduação em Oncologia. Brazil, 2012.
14. **Poster presentation** at Death Danger Inflammation and Immunity - EATI Conferences. France, 2012
15. **Poster presentation** at 36th Congress of the Brazilian Society of Immunology. Brazil, 2012.
16. **Poster presentation** at III Jornada do Programa de Pós-Graduação em Oncologia. Brazil, 2011.
17. **Poster presentation** at 4° Simpósio de Imunobiologia de Tumores da UNESP. Brazil, 2011.
18. **Short talk** at International Cell Death Society Syposium, Brazil, 2011.
19. **Short talk** at 3° Semana Acadêmica da Biomedicina and 4° Workshop em biotecnologia da UNIARARAS. Brazil, 2010.
20. **Attendance** at 2º Semana Acadêmica da Biomedicina UNESP. Brazil, 2009.
21. **Poster presentation** at 4º Congresso Científico Uniararas - 3º Congresso de Iniciação Científica PIBIC-CPNQ. Brazil, 2009.
22. **Poster presentation** at 55º Congresso Brasileiro de Genética. Brazil, 2009.
23. **Attendance** at 1º Semana Acadêmica da Biomedicina. Brazil, 2008.
24. **Poster presentation** at 3º Congresso Científico Uniararas - 2º Congresso de Iniciação Científica. Brazil, 2008.
25. **Poster presentation** at IX Simpósio Nacional de Biologia Molecular aplicada à Medicina. Brazil, 2008.
26. **Poster presentation** at The First Meeting On Anging. Brazil, 2008.
27. **Poster presentation** at VIII Workshop de Genética. 2008.
28. **Attendance** at 10° Encontro regional de Biomedicina - ERBM. Brazil, 2007.
29. **Attendance** at 2° Workshop de Biotecnologia. Brazil. 2007.
30. **Short talk**, at 2º Congresso Científico Uniararas - 1º Congresso de Iniciação Científica PIBIC - CNPQ. Brazil, 2007.
31. **Attendance** at 1º Workshop de Biotecnologia. Brazil, 2006.
32. **Attendance** at 9º Encontro Regional de Biomedicina - ERBM. Brazil, 2006.

Universidade de São Paulo
Brasil

Janus
Sistema administrativo da Pós-graduação

Faculdade de Medicina

ATESTADO

Atestamos, para os devidos fins que, até o dia 12/11/2017, o(a) senhor(a) Ruan Felipe Vieira Medrano, de número USP 7358822, cursou a(s) disciplina(s) abaixo na qualidade de aluno(a) regular do programa de pós-graduação em Oncologia.

Disciplina: Adesão Celular e Câncer
Sigla: MCM5851-4/1 **Carga Horária:** 90 **Conceito:** B **Frequência:** 90 **Créditos:** 6
Início: 15/10/2013 **Término:** 16/12/2013

Disciplina: Regulação da Resposta Imune
Sigla: BMI5881-4/2 **Carga Horária:** 120 **Conceito:** A **Frequência:** 90 **Créditos:** 8
Início: 10/03/2014 **Término:** 12/05/2014

Disciplina: Terapia Gênica do Câncer: Tecnologia e Aplicação
Sigla: MCM5907-1/2 **Carga Horária:** 120 **Conceito:** A **Frequência:** 83 **Créditos:** 8
Início: 08/09/2014 **Término:** 02/11/2014

Disciplina: Mecanismos de Ação de Agentes Imunomoduladores. "Mechanisms of Action of Immunomodulatory Agents"
Sigla: MCM5918-1/1 **Carga Horária:** 30 **Conceito:** A **Frequência:** 100 **Créditos:** 2
Início: 21/09/2015 **Término:** 27/09/2015

Documento emitido às 22:22:55 horas do dia 12/11/2017 (hora e data de Brasília).
Código de controle: W34J - ES3X - 7986 - KE7A
Código de controle válido até: 12/11/2018

Faculdade de Medicina

Aluno: Ruan Felipe Vieira Medrano, número USP 7358822

Programa: Oncologia.

Disciplina: Imunoterapia para Doenças Neoplásicas

Sigla: MCM5919-2/1 **Carga Horária:** 30 **Conceito:** A **Frequência:** 100 **Créditos:** 2

Início: 24/04/2017 **Término:** 30/04/2017

Disciplina: Desenvolvimento de Vacinas em Doenças Infecciosas

Sigla: MCM5920-2/1 **Carga Horária:** 30 **Conceito:** A **Frequência:** 100 **Créditos:** 2

Início: 08/05/2017 **Término:** 14/05/2017

Disciplina: Imunoterapia para Doenças Inflamatórias Crônicas

Sigla: MCM5921-2/1 **Carga Horária:** 30 **Conceito:** A **Frequência:** 100 **Créditos:** 2

Início: 05/06/2017 **Término:** 11/06/2017

Disciplina: Imunobiologia dos Tumores

Sigla: BMI5853-5/1 **Carga Horária:** 60 **Conceito:** A **Frequência:** 90 **Créditos:** 4

Início: 08/08/2017 **Término:** 30/08/2017

Conceito a partir de 02/01/1997:
A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.
Um(1) crédito equivale a 15 horas de atividade programada.

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Código de controle: W34J - ES3X - 7986 - KE7A

Código de controle válido até: 12/11/2018

Faculdade de Medicina

Aluno: Ruan Felipe Vieira Medrano, número USP 7358822

Programa: Oncologia.

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Código de controle válido até: 12/11/2018

WASHINGTON UNIVERSITY

School of Medicine

Department of Pathology and Immunology

REPORT

Development, Validation and Optimization of Combinatorial Cancer Immunotherapy Using Personalized Neoantigen Vaccines Together with Monoclonal Antibodies Targeting Immune Checkpoints

Ruan F. V. Medrano

Internship supervisor: Dr. Robert Schreiber

Ph.D supervisor: Dr. Bryan E. Strauss

São Paulo

2017

PROJECT ABSTRACT

Among the current strategies to use the immune system to treat cancer patients, the blockade of immune checkpoints has yielded highly encouraging results in several clinical trials. Recent work in Dr. Schreiber`s laboratory has used a combination of next generations sequencing, bioinformatics and state-of-the-art immunology techniques to shown that tumor specific T cells reactivated upon checkpoint blockade preferentially react with epitopes of mutant tumor antigens. The work in the Schreiber lab has also led to the development of distinct families of carcinogen induced mouse sarcomas that (a) are rejected spontaneously when injected into naïve syngeneic wild type mice; (b) grow progressively in wild type mice but are rejected when tumor-bearing mice are treated with either anti-CDTLA-4, anti-PD-1, or a vaccine consisting of synthetic long tumor-specific mutant neoepitope administered as mono- or combination-therapies; or (c) are not rejected when tumor bearing mice are treated with checkpoint blockade or tumor specific vaccines but whose growth can be controlled partially when mice are treated combinations of anti-CTLA-4, anti-PD-1 and/or synthetic long neoepitope vaccines. Preliminary data suggest that therapeutic control of this third group can be improved if we can improve the efficacy of our vaccination protocols. Thus in this project, we wish to formally test the hypothesis that more efficient vaccination methods would improve the therapeutic outcome of checkpoint blockade immunotherapy. For this purpose, we aim to explore different types of mutant tumor-specific antigen vaccines such as those that contain synthetic long peptides that represent additional tumor specific antigens, epitopes delivered using a double attenuated *Listeria* vaccine, DNA vaccines or as dendritic cells fed neoepitopes.

INTRODUCTION

Although the concept that the immune system can prevent and combat tumor development in an immune competent host was proposed over 100 years ago (Ehrlich 1909; BURNET 1957), it was not until recently that this concept was formally demonstrated (Shankaran, Ikeda et al. 2001). However, as a result of immune pressure on cancer development, tumor immunogenicity can sometime be negatively sculpted and as a consequence the edited tumor cells that now begin to grow progressively create a highly immunosuppressive tumor microenvironment that subverts anti-tumor immune mechanisms (Schreiber, Old et al. 2011; Vesely, Kershaw et al. 2011). This paradoxical dual host protective versus tumor promoting function of immunity has become known as cancer immunoediting and has formed the conceptual basis for much of the recent development of effective, cancer immunotherapies that are in use today.

Indeed, in a significant number of cancer patients, it is now accepted that the reestablishment of a functional anti-tumor immune response is a determining factor for a successful cancer treatment (Locher, Conforti et al. 2010). Among the current immunotherapeutic strategies, the blockade of immune checkpoints are obtaining promising result in several clinical trials (Adachi and Tamada 2015; Baksh and Weber 2015; Postow, Callahan et al. 2015).

Immune checkpoints are inhibitory pathways critical for the immune system to maintain self-tolerance and modulate the duration and amplitude of physiological immune responses in peripheral tissues (Pardoll, 2012). Tumors exploit this regulatory pathways to evade from the immune response, especially from the T lymphocytes (Cavallo, De Giovanni et al. 2011). Nevertheless, this represents a therapeutic opportunity since these pathways can be blocked by antibodies or other methods.

The use of monoclonal antibodies against CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) increased global survival of patients with advanced melanoma and became the first of its class to be approved by the FDA (US Food and Drug Administration) (Phan, Yang et al. 2003; Hodi, O'Day et al. 2010). More recently, antibody blockade of PD-1 (Programmed cell death 1) and its ligand PD-L1, is obtaining satisfactory and durable results in diverse types of cancer and has also been approved by the FDA (Brahmer, Drake et al. 2010; Hersey and Gowrishankar 2015).

As mentioned above, blockade of CTLA-4 and PD-1 pathways have yielded significant benefits for the treatment of cancer. However, only between 20-40% of patients with certain types of cancer respond to checkpoint blockade immunotherapy and thus there is a critical medical need to develop methods to improve responsiveness

in other nonresponding cancer patients and in patients with different forms of cancers that show limited responses to checkpoint antibodies. An important insight into why some patients respond to checkpoint blockade while others do not was recently made in Dr. Schreiber's lab. (Gubin, Zhang et al. 2014) (Yadav, Jhunjhunwala et al. 2014) (Tran, Turcotte et al. 2014). In this work, it was used genomics and bioinformatics methods to identify, in progressively growing sarcomas, two mutant antigens (mutant Lama4 and mutant Alg8) as targets of a specific T cell response, thus showing that mutant antigens are important targets of T cells reactivated by checkpoint blockade therapy.

Furthermore, it was generated a Synthetic Long Peptide vaccine (SLP) based on the mutant tumor antigens and asked if its use in therapeutic vaccination could protect against tumor outgrowth. Remarkably, 90% of the vaccinated mice rejected their tumors in comparison with only 10% of tumor bearing mice treated with irrelevant SLP plus Poly (I:C) **(Figure 1)**. The strategy used in this work, which is identification of immunogenic mutant antigens and creation of a tumor-specific vaccine, can be used for further development of personalized cancer vaccines (Gubin, Zhang et al. 2014).

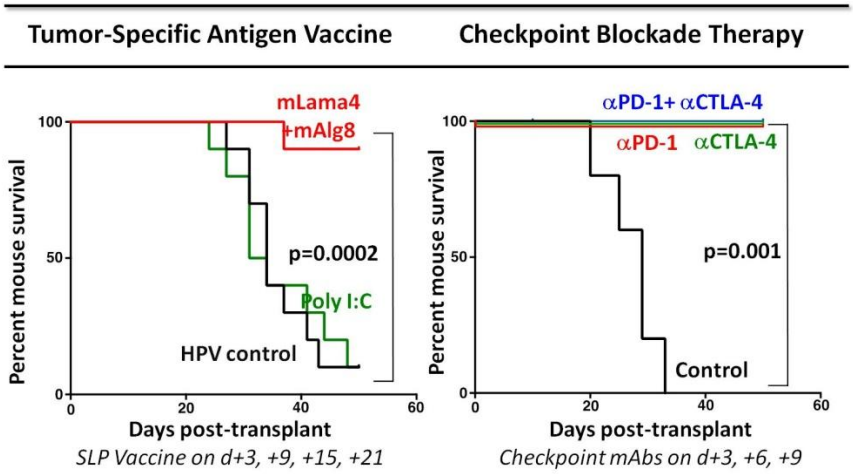

Figure 2. Comparable Efficacies between Checkpoint Blockade and Personalized Cancer Vaccines (Gubin, Zhang et. 2014)

SLP vaccines are taken up by APCs, where they are presented by both MHC I and II molecules (Yamada, Sasada et al. 2013). Yet, inclusion of adjuvants has been shown to be crucial, as peptides by themselves are poorly immunogenic or can even induce tolerance (Arens, van Hall et al. 2013). Conjugation of Toll Like Receptor (TLR) ligands with peptide antigens improves trafficking and intracellular processing leading to optimal antigen presentation. Indeed, the SLP vaccine used for the delivery of mutants Lama4 and Alg8 was combined with poly (I:C), a TLR 3 agonist (Gubin, Zhang et al. 2014).

In order for the SLP vaccine to be effective, vaccinations must be initiated no later than day 4 after tumor inoculation. If started on day 5, the effects are only observed in 50% of the mice and almost not at all if the treatment is made on day 6. Interestingly, by combining checkpoint blockade (anti PD-1) with the SLP vaccine, effectiveness is again observed, even if vaccination starts at day 6 or 7 **(Figure 2)** (unpublished data).

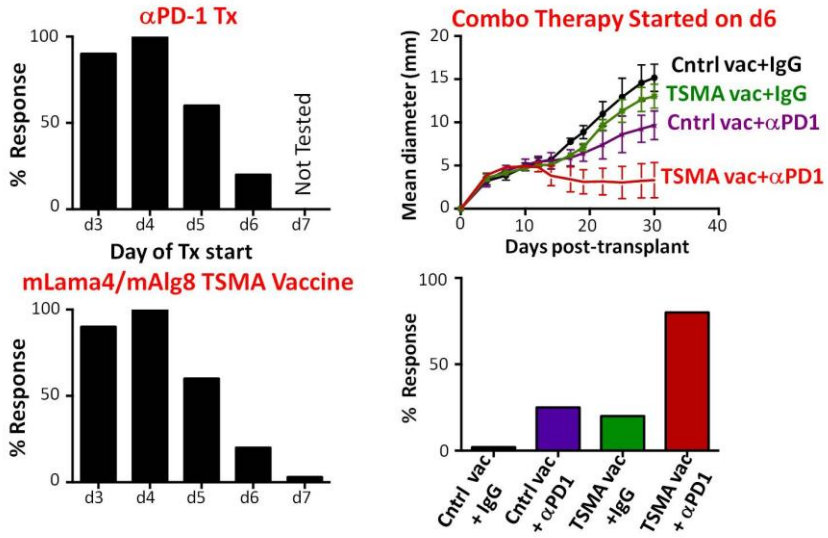

Figure 3. Tumor Specific Mutant Neoantigen Vaccine Extends the Anti-PD-1 Therapeutic Window for T3 (unpublished data).

This data suggests that during tumor progression, immune suppressive mechanisms might be impairing the anti-tumor response elicited by the SLP vaccine. Therefore, for the development of a truly effective immunotherapy that associates antigen-specific vaccines and checkpoint blockade, more efficient vaccination methods will be investigated in this project.

Among the current vaccine vectors that can be used for such purpose, we are going to explore the following four: SLP vaccines containing additional predicted neoantigens from the tumors, double attenuated *Listeria* vaccines, DNA vaccine and dendritic cells fed with peptides. We plan to use these different vaccination methods on (a) tumors like the T3 sarcoma that can be controlled by either checkpoint blockade alone or SLP vaccines alone when administered early and can be controlled by the combination of these two when administered when tumors reach approximately 1 cm in diameter; and (b) MCA sarcomas such as F279 which display lower immunogenicity because they are not controlled by checkpoint blocking mAbs or SLP vaccines when administered at day 3 after tumor injection and which display only partial responses even when checkpoint mAbs and SLP vaccines are used in combination.

Listeria monocytogenes (LM) are intracellular bacteria that can be phagocytosed by APCs, macrophages and DCs, in the spleen and Kupffer cells in liver. Once inside

the cells, the bacteria perforates the phagosome and secretes proteins that are degraded into peptides to be presented by both class I and II MHC molecules. Because of this, LM can induce a $CD4^+$ and $CD8^+$ antigen-specific immune response (Yang, Hou et al. 2014).

However, attenuation of LM virulence is needed to assure safety of the procedures. The strain we are going to use was bioengineered by Aduros Biotech. Their approach uses a live, attenuated, double-deleted *Listeria* strain in which the virulence genes, actA and inlB, have been deleted. Further modifications made it possible to express different tumor antigen genes. For example, the CRS-207 pancreatic cancer vaccine, considered by the FDA as a breakthrough when applied with GVAX, is engineered to express human mesothelin (a common pancreatic cancer antigen) (Sinha 2014).

DNA vaccines are considered to be a simple, safe and attractive immunotherapeutic approach. The results of various clinical trials have demonstrated that vaccinated patients do not generate severe adverse effects. DNA vaccines can be designed to encode different antigens and immunomodulatory molecules (Yang, Jeang et al. 2014). A recent example is the DNA vaccine that encodes mammaglobin-A (MAM-A), which is expressed in 40% to 80% of breast cancers. In a phase I clinical trial, vaccination with MAM-A DNA vaccine resulted in an antigen-specific $CD8^+$ T-cell immune response and an increase in IFN-γ secreting cells. Moreover, no significant adverse effects were observed and preliminary data suggests improvement of progression free survival (Tiriveedhi, Tucker et al. 2014).

DCs can be considered as an ideal adjuvant in peptides cancer vaccines as they are extremely efficient in presenting antigens and in stimulating a T cell response (Carreno, Magrini et al. 2015). Several clinical trials have used autologous DCs and tumor-associated antigen peptides to unleash an antitumor response in cancer patients (Yamada, Sasada et al. 2013). A recent advance in the field has identified mutant antigens from melanoma patients to generate a personalized DC vaccine with tumor-specific peptides. Results of this phase I clinical trial indicates that DC vaccination loaded with tumor-derived mutant peptides to be safe and showed an increase of antigenic breadth and clonal diversity of antitumor immunity (Carreno, Magrini et al. 2015).

Based on the above arguments, we hypothesize that further refinements of the method used for delivery of the mutant tumor antigens could improve the therapeutic outcome of cancer vaccines associated with checkpoint blockade immunotherapy.

OBJECTIVES

Main objective: Evaluate methods for the delivery of mutant tumor antigens in order to develop a cancer-specific vaccine in association with checkpoint blockade immunotherapy.

Specific objectives:

1. Vaccinate, individually, with each vector (double attenuated *Listeria*, DNA and dendritic cells vaccines) in association with/without checkpoint blockade (CTLA4, PD-1 or CTLA-4 + PD-1) and compare the therapeutic effect with the SLP vaccine plus checkpoint blockade.
2. Analyze the immune response, in lymphoid organs and in the tumor, of the best vector (determined above) in association with/without checkpoint blockade.

MATERIALS AND METHODS

Cell culture and tumor transplantation

Cell culture conditions and tumor transplantation methods were performed according Gubin, et al, 2014. Briefly, the MCA-induced sarcoma tumor line (generated in male 129S6 strain wild-type and originally described in Shankaran, et al 2001) named d42m1-T3 (hereinafter, just T3) and was maintained *in vitro* in RPMI media (Hyclone) supplemented with 10% FCS (Hyclone), washed two times, resuspended at a density of 6.67×10^6 viable cells/ml in endotoxin-free PBS and then 150 µl were injected subcutaneously (s.c) into the left flank of syngeneic mice. Tumor progression was followed by caliper measurements and expressed as the average of two diameters.

129S6 wild type and *Rag2*$^{-/-}$ mice (male, 8 week old) were purchased from Taconic Farms and were bred in a specific-pathogen free animal facility. Experiments were made performed in accordance with procedures approved by the animal studies committee of Washington University in St Louis.

Antibody staining and flow cytometry

Fluorescently conjugated antibodies against CD45, Thy1.2, CD4, CD8alpha, PDL-1, MHC-II, CD80, CD40, SIRP-alpha, CD24, CD103, F4/80 were purchased from BioLegend while anti-FOXP3 was obtained from BdBiosciences. Fc block (anti-CD16/32) was purchased from BD Bioscience. Cells were stained (20 min at 4 °C) with 500 ng of Fc block (for 5 min, room temperature) and then stained in 100 µl solution of staining buffer (PBS with 2% FCS and 0.05% NaN_3 (Sigma) with 1:200 of each antibody. Flow cytometry was performed on the FACSCalibur (BD Biosciences) and analyzed using the FlowJo software (TreeStar).

Tetramer staining

H-2Kb or H-2Db tetramers conjugated to phycoerythrin (PE) or Allophycocyanin (APC) were produced in house, prepared with mutant or irrelevant short peptides and staining was done as described in Gubin, et al. 2014.

Anti-PD-1 Checkpoint blockade immunotherapy

Mice will were treated (i.p) with 200 mg of anti-PD-1 (rat chimaeric murine IgG1 Antibody, clone 4H2, Leinco Technologies) on days 3, 6, and 9 post-tumor transplant.

SLP therapeutic vaccination

Mice were vaccinated (s.c), on days 3, 9, 15 after tumor cell transplantation, with the mLama4 or mAlg8 SLP peptides (50 μg for each or as otherwise explained) with the adjuvant poly(I:C) (100 μg, unless otherwise explained, Invivogen). And as a vaccine control, mice were vaccinated with a human papilloma virus (HPV) SLP with poly(I:C). The mLama4 peptide sequence used was QKISFFDGFEVGFNFRTLQPNGLLFYYT (CD8 epitope underlined) and for the mAlg8 peptide sequence was AVGITYTWTRLYASVLTGSLV (CD8 epitope underlined).

6H3mLama4/GFP-MILD and 6H3mLama4/GFP-HIGH cell sorting, and qPCR for mLama4 and mAlg8

6H3 cells were generated from the d42m1-T3 cell line that had the mutant allele of the Lama4 gene repaired to its wild type form with the CRISPR/Cas9 Technology (data not shown). Retrovirus vector encoding both the mutant Lama4 allele and the GFP reporter gene was produced in the lab by transfecting phoenix cell and all supernatant was used in combination to transduce the 6H3 cells. Cell sorting was realized in a LSR Fortessa (BD Biosciences) flow cytometer based on the GFP intensity and was repeated three times to obtain the 6H3mLama4/GFP-MILD and 6H3mLama4/GFP-HIGH cell lines with 99% of purity.

qPCR was performed as described in Gubin, et. al. 2014. Primers used were: tmutant Lama4, forward primer (5'-GGATGCCCAGAGGACTCTCTG-3') and reverse primer (5'-GTAATGTTCGGAAATTGAAGCCTA-3'). For detection of mutant Alg8, forward primer (5'-TCCCGTTTACCTCCTGGAAGC-3') and reverse primer (5'-AGCATACAGCCTGGTCCAGGT-3').

Nanoparticle immunization and IFN-γ ELISPOT

Neoantigen-cage fusion nanoparticles were kindly provided by the Neil King Lab (Washington University in Seattle, USA) and were described in Hsia, et al. 2016.

Neo-antigens have a 25 nanometer icosahedral nanocage with 60 copies of the epitope attached by genetic fusion, confirmed by Mass spectrometry.

Naïve 129S6 mice were immunized (i.p or s.c), on days -14 and -7, with Poly IC (100µg) as adjuvant and with the mSpectrin-β2 Nanoparticles (10µg) or mSpectrin-β2 short peptide (10µg). Seven days after the last immunization, spleens were harvest, splenocytes isolated, cultured *in vitro* with 1µM of mutant or wild-type peptides for 20h in a IFN-γ ELISPOT pre-coated 96 wells plate (Millipore). Plates were analyzed on an ImmunoSpot reader and reagents for ELISPOT were purchased from Mabtech.

Bone marrow derived CD103$^+$ dendritic cells

Dendritic cells (DC) were obtained following the protocol described in Mayer, et el. 2014. In detail, bone-marrow cells were harvested from the mice femur and tibia and $15x10^6$ cells were cultured in 10cm petri dishes with 10% FBS RPMI medium, 5-10% of FLT3 conditioned media and 5ng/ml of GM-CSF for 9 days. Subsequently, cells were equally divided and replated into two 10 cm petri dish with the same media and combination of cytokines for more 5 days, obtaining on average $4x10^7$ cells. Differentiation was confirmed by flow cytometry and maturation treatment was made with the combination of Poly IC 50 µg/mL and Pam3CSK4 1µg/mL for 24 hours. Supernatant was collected and IL-12p70 production analyzed with Mouse IL-12 (p70) ELISA MAX™ Capture Antibody (200X) kit (Biolegend).

DATA PRESENTED HERE

Here, we report our efforts in developing platforms for personalized cancer vaccines based on the delivery of the following tumor neoantigens: mLama4 and mAlg8 specific for the MCA sarcoma cell line d42m1-T3 (hereinafter, just T3), as well as the mSpectrinB2 neoantigen specific for another MCA sarcoma cell line, d42m1-T9 (hereinafter, just T9).

As proposed, we explored three distinct strategies: (i) synthetic long peptides (SLP), (ii) neoantigen-cage fusion nanoparticles and (iii) bone marrow-derived CD103$^+$ dendritic cells. While working with the SLP vaccine we faced optimization issues that led us to investigate the influence of the target antigen stability, CD4 and CD8 epitopes preset in the mLama4 SLP and the ratio between the amounts of poly(I:C) and peptide.

Finally, we also show that immunization with the neoantigen-cage fusion nanoparticles can induce an antigen specific CD8$^+$ T cell responses and characterize bone marrow derived CD103$^+$ dendritic cells. It is important to point that the Listeria vaccine and the DNA vaccine originally proposed in the project were substituted for the

nanoparticle vaccine, duo to novelty opportunity presented on working with the nanoparticles.

RESULTS AND DISCUSSION

mLama4 and mAlg8 SLP vaccine

In the first set of experiments we decided to optimize the mLama4 and mAlg8 SLP vaccine, so in this way we would be sure that it was in its best performance and thus could be accurately compared with other more sophisticated methods of vaccination. We sought to maintain the dose of 100 µg of Poly (I:C) and just titrate the amount of mLama4 and mAl8 SLPs, starting with the standard dose of 50 µg of each SLP and decreasing by jumps of two till 1.25 µg. Vaccinations were performed at the same side where the T3 tumor cells were transplanted and started on day 3, being repeated three times, every 6 days. Unexpectedly, results were lacking consistency among the different experiments and, as indicated in **figure 3a,** mice vaccinated with the mLama4 and mAlg8 SLP presented just a decrease in tumor progression, not a complete tumor regression. Attempts to work with a superior quality of poly (I:C), the endotoxin free Poly (I:C) vaccigrade, side of vaccine (same side of the tumor vs opposite side) and day of vaccine (day +3 or +4) were made and no improvement could be observed, leading us to ask if the mLama4 antigen level had in the T3 tumor line **(Figure 3b).** Indeed, by comparing the current batch of cells (2015) with an older batch (2013) and a sub clone of the T3 cell line we could observe that the mLama4 expression levels were varying drastically just in the 2015 batch of cells. Furthermore, this variability reflected on the secretion of IFN-γ by a CTL (cytolytic t cell line) clone specific for mLama4, as shown by the error bar of the 2015 batch **(Figure 3c).** Interestingly, both the 2013 T3 batch and the T3.1 sub clone were presenting to have a more consistent expression of mLama4 and thus, in continuation, we decided to use the T3.1 tumor line, assuming that since it was a sub clone the heterogeneity of target antigens would be reduced and benefit the vaccine result.

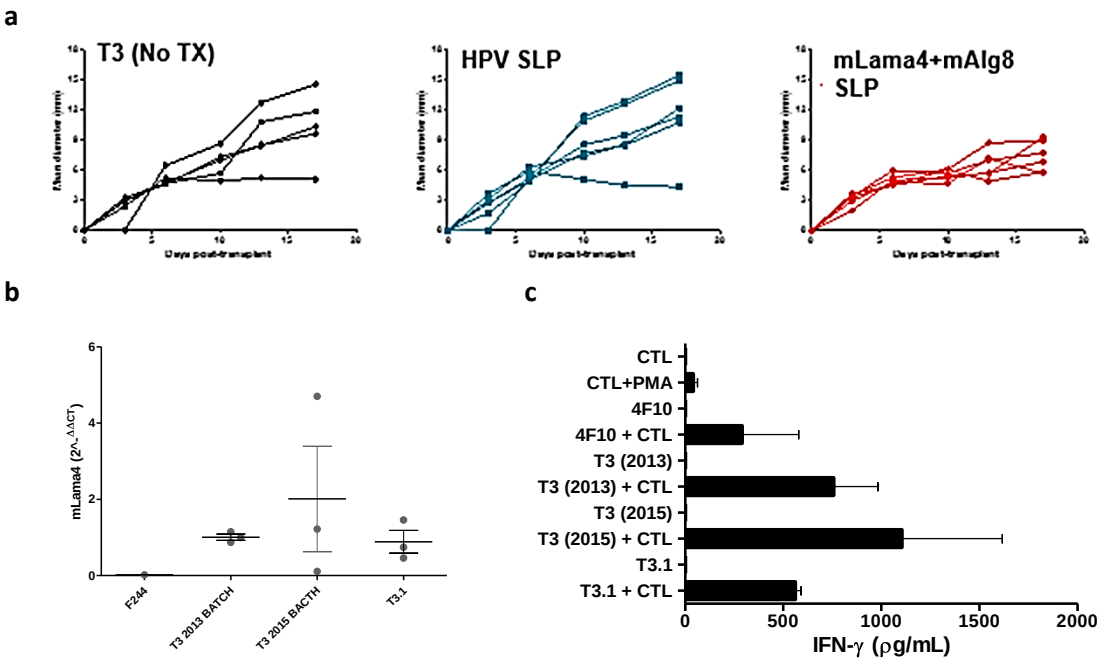

Figure 3. (a) mLama4 and mAlg8 SLP vaccine in the batch of T3 cells from 2015. Mice harboring T3 tumors were vaccinated with mLama4 and mAlg8 SLPs or HPV SLPs and tumor growth followed up. N=5 in all groups **(b)** Comparison of mLama4 expression between batches of T3 cell line. Cells from T3 from 2013 or 2015 batches, the T3.1 subclone and F244 (unrelated tumor) were harvested, RNA extracted and qPCR performed. Three independent passages were analyzed **(c)** CTL IFN-gamma stimulation comparing different batches of T3. Supernatant from **c**ells from T3 from 2013 or 2015 batches, the T3.1 sub clone and F244 (unrelated tumor) were co-cultured with a CTL clone specific for the mLama4 antigen.

However, when using the T3.1 tumor line we could observe complete tumor regression upon the vaccination with the mLama4 and mAlg8 SLPs, yet control groups also showed tumors that regressed **(Figure 4a)**. Interestingly, these tumors presented a high percentage of infiltrating CD8+ T cells (60%) and low FOXP3^{+} CD4+ T cells (22%), percentages that seemed unusually different from what we had being observing in tumors at day 25 **(Figure 4b and 4c)**.

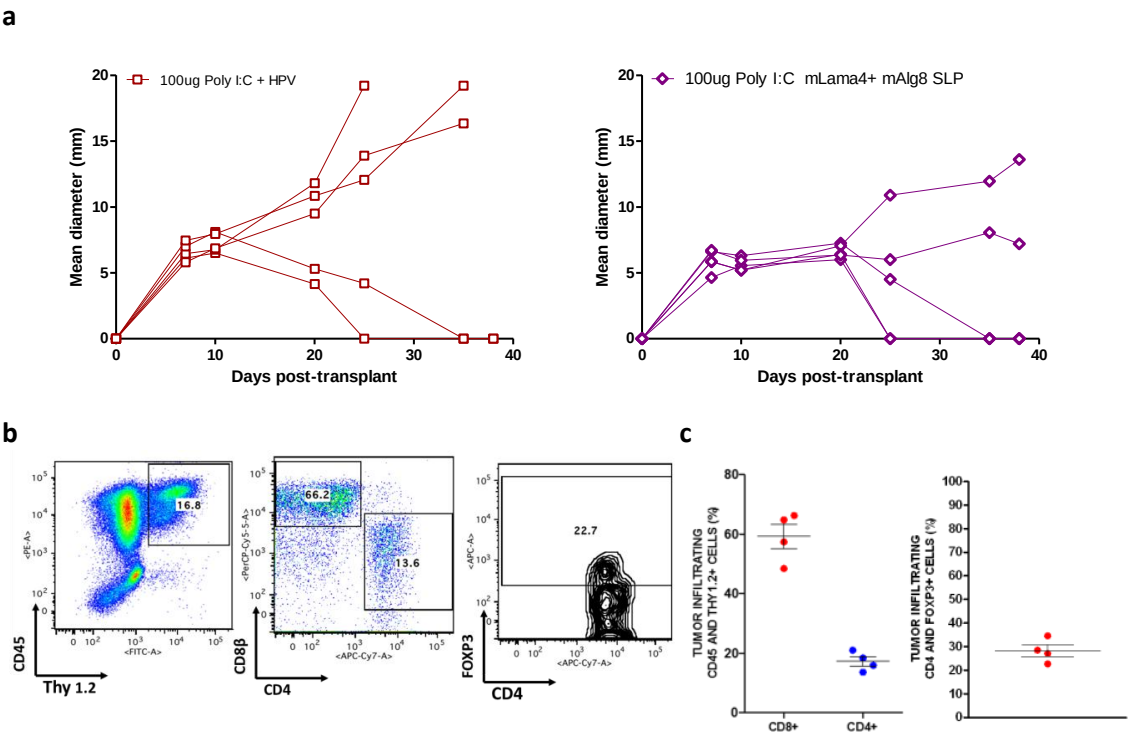

Figure 4. (a) mLama4 and mAlg8 SLP vaccine in the T3.1 tumors. Mice harboring T3.1 tumors were vaccinated with mLama4 and mAlg8 SLPs or HPV SLPs and tumor growth followed up. N=5 in all groups **(b and c) Tumor infiltrating T cells from the mLama4 and mAlg8 group**. CD45 cells were gated after being positives for Thy1.2, CD8β, CD4 and FOXp3 Comparison of mLama4 expression between batches of T3 cell line. Tumors were harvest at day 25 and n = 4.

These observations encouraged us to investigate if this tumor rejection was mediated by the immune system and to do so, we compared the immunogenicity of T3.1 and T3 (2013) tumors by injecting these cells in immunocompetent and immunodeficient hosts. As expected, half of T3.1 tumors were spontaneously rejected when inoculated in immunocompetent hosts, whereas T3 tumors showed a consistent progressor growth rate **(Figure 5a).** In support of this evidence we also analyzed infiltration of antigen-specific CD8+ T cells and T3.1 tumors had a higher percentage of mLama4 CD8+ T cells (15%) when compared to T3 tumors from the batch of 2013 (6%), possibly explaining why these were being spontaneously rejected **(Figure 5b)**.

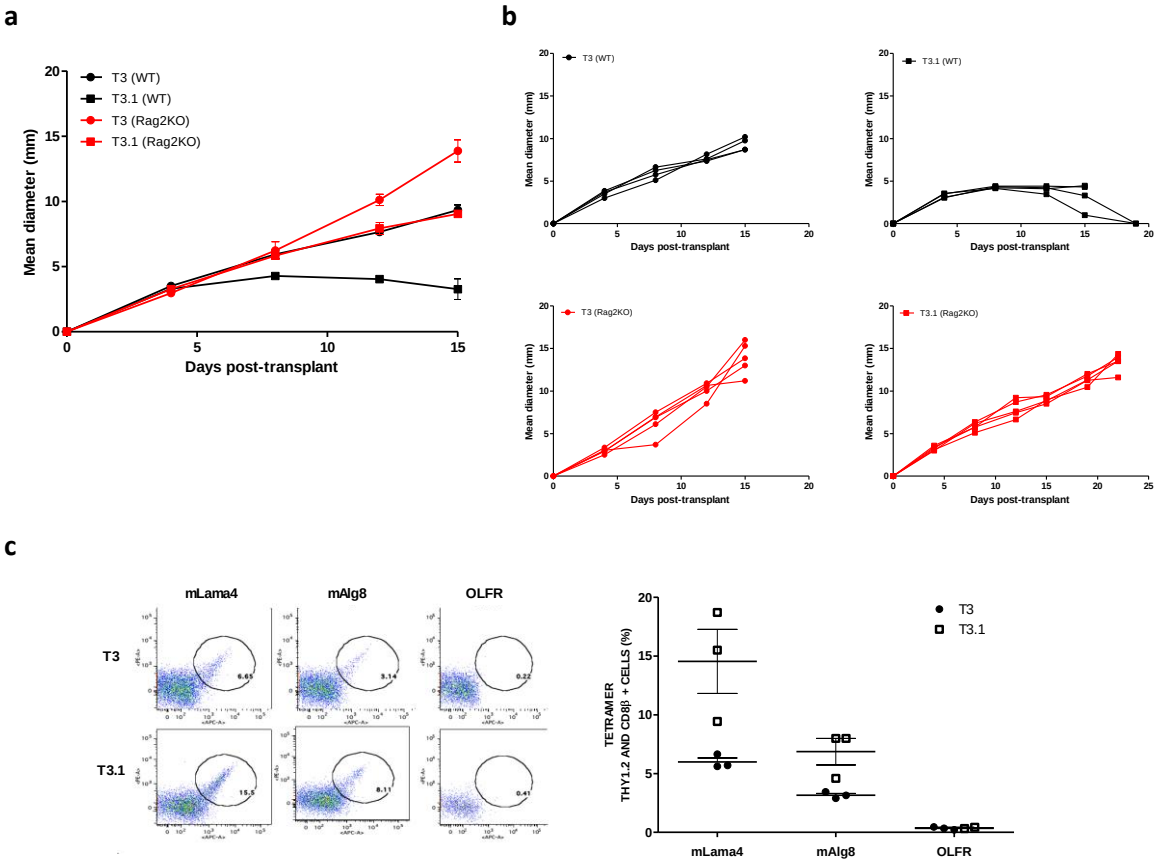

Figure 5. T3 and T3.1 distinct immunogenicities. (a) averages (b) individual tumor growth fromT3 and T3.1 cells inoculated into WT immunocompetent mice (black line) or Rag2KO immunodeficient (red line). N=5 in all groups**. (c) mLama4 and mAlg8 tumor infiltrating CD8+ T cells.** T cells were harvest from tumors t day 15 and stained for Thy1.2, CD8+ and tetramer stained for mLama4, mAlg8 or OLFR irrelevant peptide. n = 3.

Next, we decided to work with the T3 batch of 2013 and once again attempt to titrate separately the amount of SLP (from 50 to 6,25 µg) and Poly (I:C) (from 60 to 5µg), while other vaccine parameters were kept the same. As observed in **figure 6a**, reduction in the amount of SLP up to 12.5µg greatly improved the vaccine result when compared to dose of 50 µg. However, full vaccine potential could only be observed when SLP amount was maintained at 50 µg and Poly (I:C) reduced from 100 to 60µg **(Figure 6b)**, thus showing that by reducing the amount of Poly (I:C) we could obtain better results. We hypothesize that this could also be a timing issue. Whereas type I interferons induced by Poly (I:C) is needed to activate the DC compartment, but its prolonged expression by higher doses of Poly (I:C) can be profoundly antiproliferative to T cells.

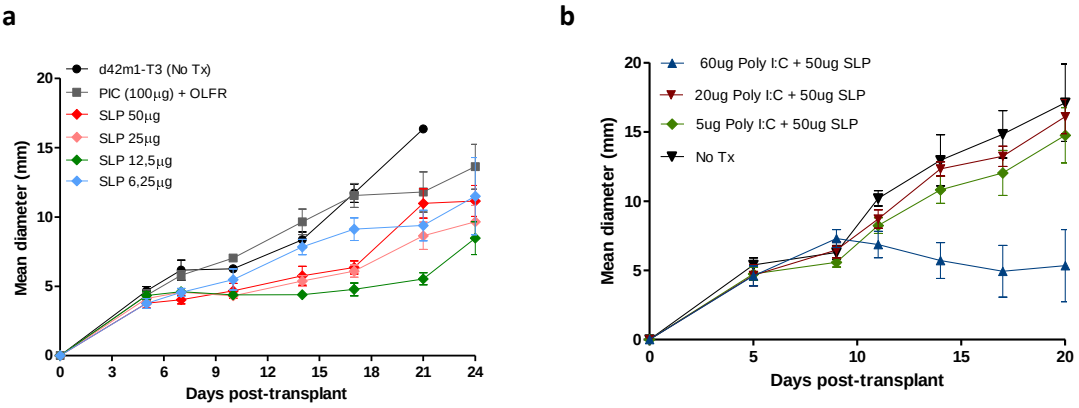

Figure 6. mLama4 and mAlg8 SLP vaccine with the batch of T3 from 2013. **(a)** Mice harboring T3 tumors were vaccinated with decreasing amounts of mLama4 and mAlg8 SLPs (50 to 6,25 µg) or HPV SLP (50 µg) keeping the same amount of poly I:C (100 µg). n=5 in all groups **(b)** Mice harboring T3 tumors were vaccinated with the same amount of mLama4 and mAlg8 SLPs (50µg) or HPV SLP (50 µg) and decreasing amounts of Poly I:C (60 to 5 µg)v. N=5 in all groups

Another possibility that is being investigated in the laboratory is related to the CD4 epitope present in the mLama4 SLP. While the CD8 minimal epitope is located where the V to G mutation is located, the CD4 minimal epitope doesn't harbor this mutation and thus immunization with either the mutant peptide or the wild type peptide can stimulate a CD4 response independently of the mutation, indicating that it is a self-epitope. Moreover, when using a mLama4 SLP that has a disrupted CD4 epitope (reduces the $CD4^+$ T cell responses) and still has the CD8 epitope, vaccination results to a superior killing capacity of $CD8^+$ T cells, indicating that CD4 responses are hampering CTLs, and thus this self-epitope is in fact a T reg epitope (data not shown).

The last hypothesis investigated in this report was related to the stability of the target antigen, mLama4. We thought that since levels of mLama4 were showing to be vary among the three batches of T3, if we could obtain a T3 line that had mLama4 more tightly regulated and stable, then it could result in superior tumor control for the SLP vaccine. In order to test this question, we took advantage of a T3 derived cell line called 6H3 that had the mutant allele of mLama4 restored by CRISPR/Cas9 to its wild type form and used a lentiviral vector to stably express again the mLama4 allele. As shown in **figure 7a**, trough the GFP reporter expression we were able to obtain two distinct populations that were successfully transduced, named 6H3 GFP-MILD and 6H3 GFP-HIGH, and indeed by qPCR analysis expressed 10 and 5 times more mLama 4 than regular T3, respectively **(Figure 7b).** Curiously, when 6H3 mLama 4 GFP-MILD cells was inoculated into wild type mice, most of its tumors were immune rejected, indicating that a neoantigen can become a rejection antigen by modulating its expression levels **(Figure 7c)**. Furthermore, this distinct antigen levels also reflected on the percentage of antigen-specific $CD8_+$ T cells, since the 6H3 GFP-MILD presented an impressive 25%

of $CD8^+$ T cells specific for mLama4 and the 6H3 GFP-HIGH 10% **(Figure 7d).** Finally, using the 6H3 GFP-HIGH that stably progress into wild type mice and the standard vaccine protocol (50 µg of each SLP and 100 µg poly (I:C), we could observe a potent tumor rejection upon vaccination the mLama4 and mAlg8 SLPs, indicating that the stability of the target antigen is indeed a critical factor for the vaccine outcome **(Figure 7e)**.

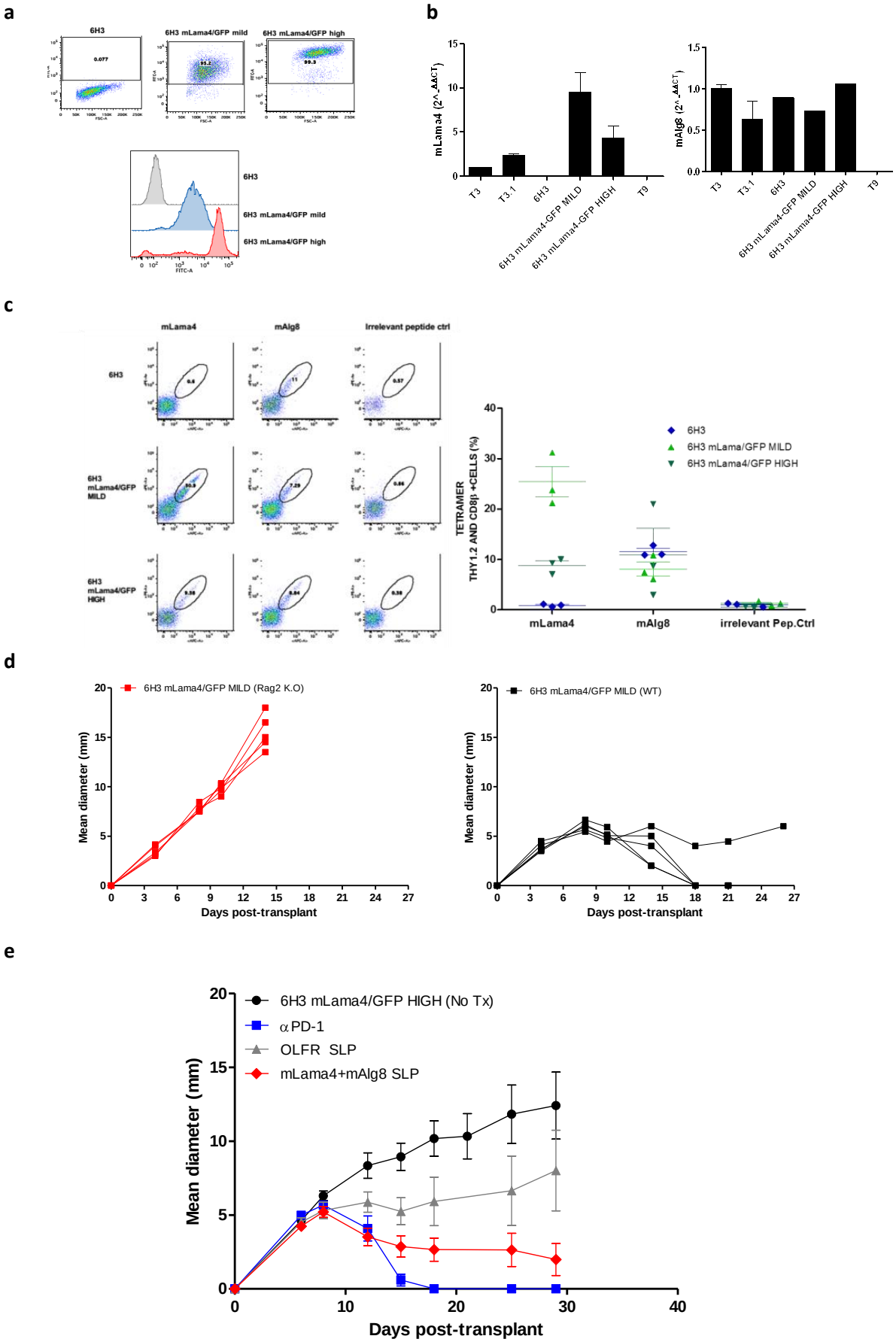

Figure 7. (a) Reestablishment of the mutant Lama4 allele in the 6H3 cell line. 6H3 cells were transduced and cell sorted based on their GFP expression. **(b)** mLama4 and mAlg8 qPCR analysis after the second round of cell sorting. **(c).** mLama4 and mAlg8 tumor infiltrating CD8+ T **cells**. 6H3, 6H3 mLama4-GFP MILD, 6H3 mLama4-GFP HIGH cells were inoculated in WT mice and 15 days tumors

were harvest and T cells analyzed according their specificity to mLama4 and mAlg8 antigens. **(d)** 6H3 mLama4-GFP MILD cells were inoculated into WT (black) or Rag2 KO mice (red) and tumor growth followed. N= 4. **(e).** Mice harboring 6H3 mLama4-GFP HIGH tumors were vaccinated with mLama4 and mAlg8 SLPs (50µg) and Poly I:C (100 µg) or with HPV SLP vaccine.

mSpectrin-β2 neoantigen-cage fusion nanoparticles

For the delivery of tumor-specific neoantigens in a nanoparticle platform we used neoantigen-cage fusion nanoparticles that were produced in the Neil King Lab (Washington University in Seattle). These nanoparticles were made by using a macromolecular design originally designed to predicted protein natural structures with an atomic-level of accuracy and are constituted by self-assembling trimeric scaffold proteins that arrange themselves into a high order symmetrical icosahedral architecture with size and molecular weights comparable to small viruses. Each particle has a 25 nanometre icosahedral nanocage with 60 copies of the antigen epitope attached to it by genetic fusion (confirmed by mass spectrometry and SDS desnaturation gel, data not shown).

Using nanoparticles with the mSpectrin-β2 CD8 epitope, on our first experiment we sought to investigate which immunization route (subcutaneous vs intra-peritoneal) would produce better immune responses, so we immunized naïve mice two times, every 7 days, with mSpcetrinβ2 nanoparticles (10µg) and Poly IC and compared the results with a short peptide immunization (10µg, s.c) encoding for the same antigen sequence. Remarkably, immunization i.p was the one that provided the best results and this was even superior than the short peptides immunization **(Figure 8a).** Moreover, we were also able to confirm that i.p immunization provided superior percentages of $CD8^+$ T cells specific for mSpectrinβ2 antigen than the other groups **(Figure 8b).**

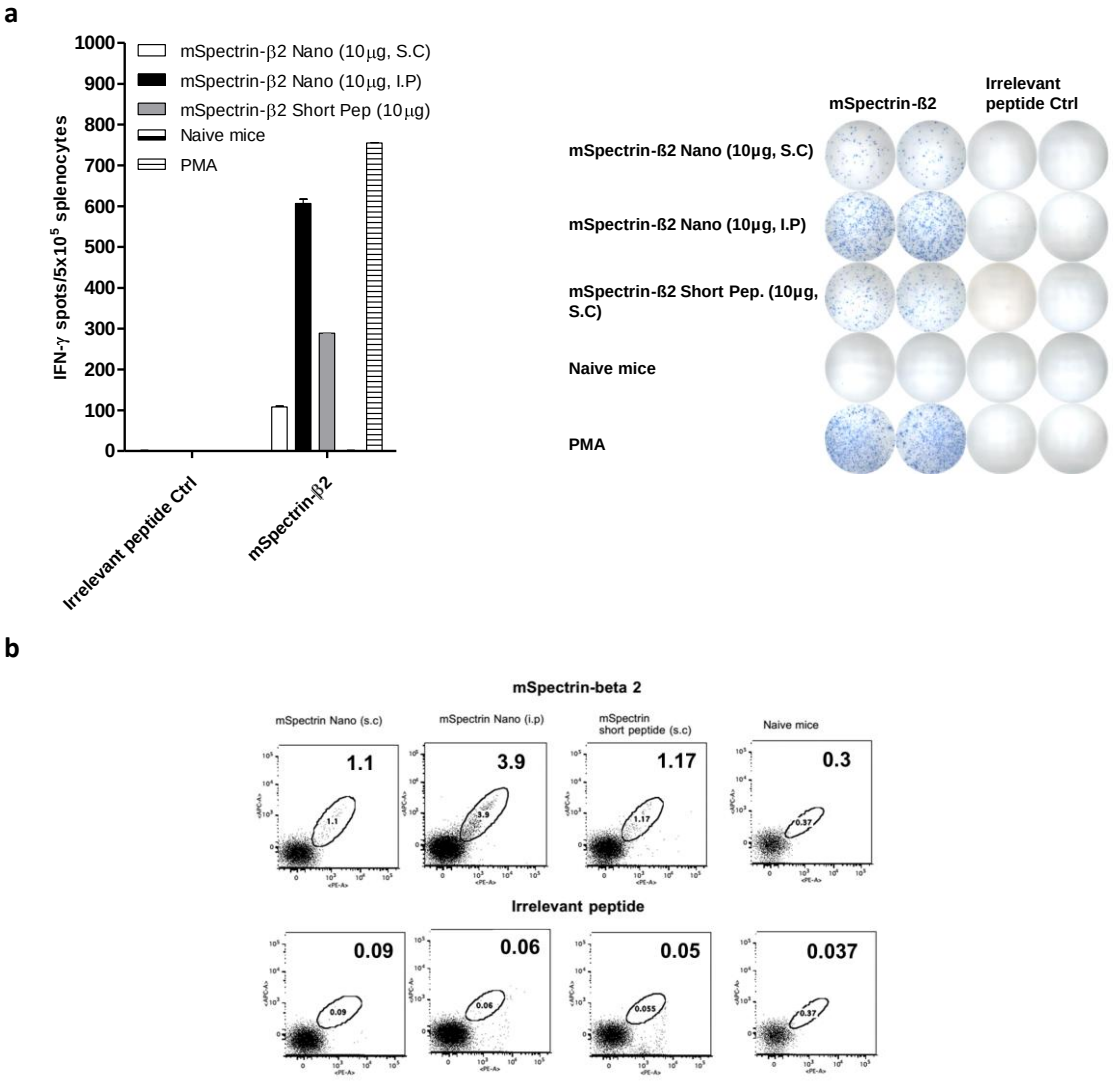

Figure 8. Immunization with mSpectrin-β2 neoantigen-cage fusion nanoparticles. (a) Naïve mice were immunized (s.c or ip) two times, every 7 days, with mSpcetrinβ2 nanoparticles (10µg) and Poly IC or with a short peptide immunization (10µg, s.c) encoding for the same antigen sequence and spleens were harvested for a IFN-gamma ELISPOT. **(b).** Spleenocytes obtained from the same experiment were also tetramer stained for mSpectrin-β2. n=2 per group.

Bone marrow derived CD103$^+$ dendritic cells

So far, the most studied method to differentiate murine bone marrow cells into dendritic cells is based on the use of medium containing GM-CSF, resulting mostly in monocyte-derived DCs. Alternatively, use of the hematopoietic cytokine FLT3L results in a mixed population of plasmacitoid DCs and conventional DCs that are further subdivided on CD11b and CD24 expression, because CD8α is absent and CD103 only variably expressed on Clec9a-expressing CD11b DCs. However, as described by Mayer, et al. (2014) culture with both GM-CSF and FLT3L for 16 days generates almost exclusively CD103$^+$ cDCs that resemble tissue-resident CD103$^+$ DCs in their phenotype, transcription factor BAFT3 dependency, expression of signature genes and function. As previously described by other authors, CD103$^+$ dendritic cells are the only antigen presenting cells capable of transport intact antigens to the lymph nodes and

prime tumor-specific $CD8^+$ T cells. Furthermore, $CD103^+$ DCs have also been shown to be required to promote immune mediated tumor rejection and anti-tumoral effects of D-L1 checkpoint blockade (Mayer, 2014).

So, with this information in mind we decided to employed the Mayer method to develop bone marrow derived CD103 dendritic cells for the delivery of neoantigens and as shown in **figure 9a,** we could successfully reproduced this method obtaining after 16 days, almost $4x10^7$ of viable cells that were 88% positive for CD11C and of those 52-60% being positive for CD103. Furthermore, as required, these cells were also CD24 positive and negative for CD8α and SIRPα. As method for maturation, we evaluate different combination of toll like receptors (TLR) agonist and the combination of poly (I:C) (TLR-3, 50 μg/mL), with Pam3Cyk (TLR1 and TLR2, 1 μg/mL), greatly enhanced expression of CD40, CD80 and MHC—II and most importantly, just upon the combined treatment, dendritic cells produced IL-12p70, a critical cytokine for the induction of a cytotoxicity CD8 mediated immune repose **(Figure 9b).**

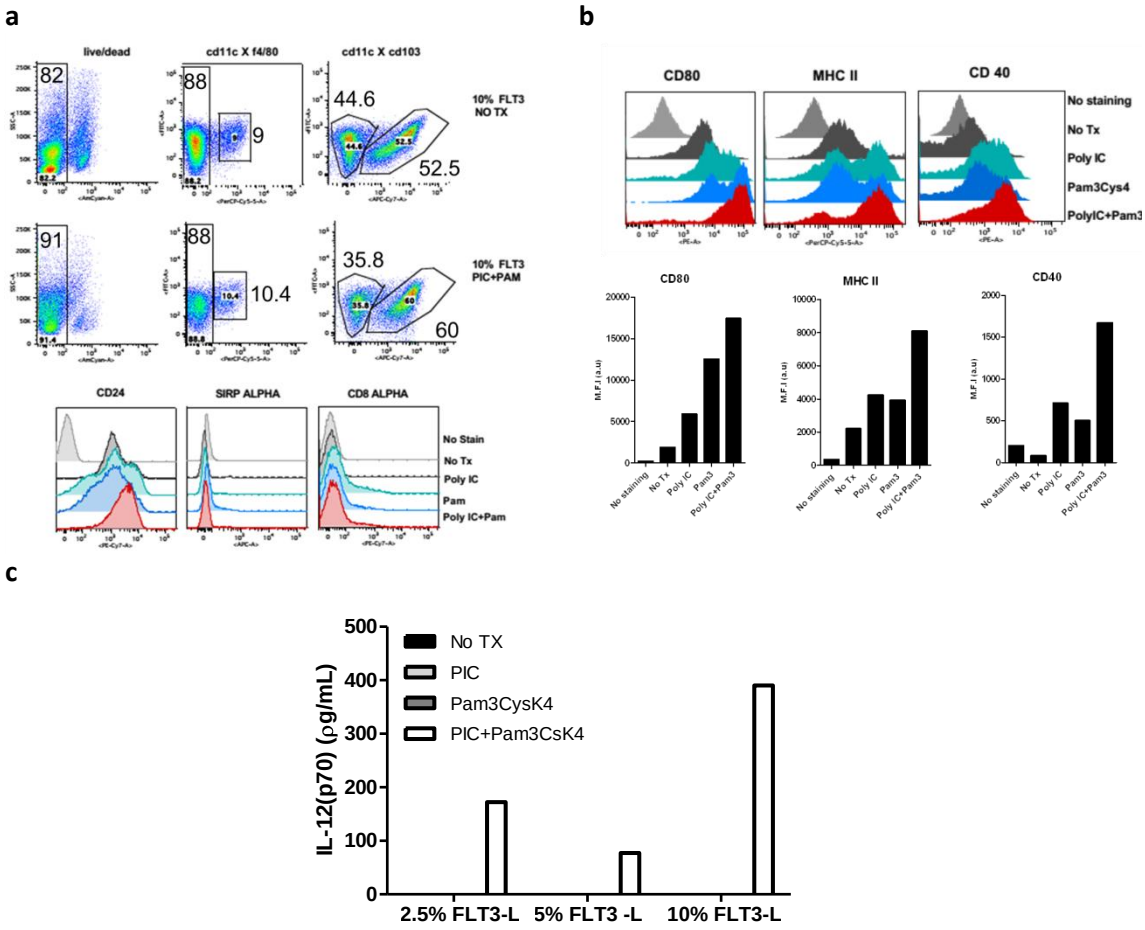

Figure 9. Bone marrow derived CD103+ dendritic cells. Bone-marrow cells were cultured with 5-10% of FLT3 conditioned media and 5ng/ml of GM-CSF for 16 days. Subsequently, cells were treated with poly (I:C) (50 μg/mL) and with Pam3CsK4 (1 μg/mL) for 24 hours, analyzed for differentiation markers **(a)** (CD11C, CD103, CD24, SIRP-ALPHA, CD-ALPHA), maturation markers **(b)** (CD80, CD40, MHC-II) and supernatant collect for IL-12p70 ELISA **(c).**

PERSPECTIVES

Although the SLP vaccine optimization took us more time than originally expected, through all of the changes on the protocol we were able to study the impact of: heterogeneity of the cell line, stability of target antigen, ration between the adjuvant and amount of SLP, route of immunization and most importantly and, most importantly, consider the requirements of an ideal neoantigen based vaccine. As long as for the other platforms, we didn't get to evaluate the use of the nanoparticles and $CD103^+$ dendritic cells in a therapeutic vaccine setting, but certainly data presented here warrants further investigation for the development of this novel neoantigen delivery platforms.

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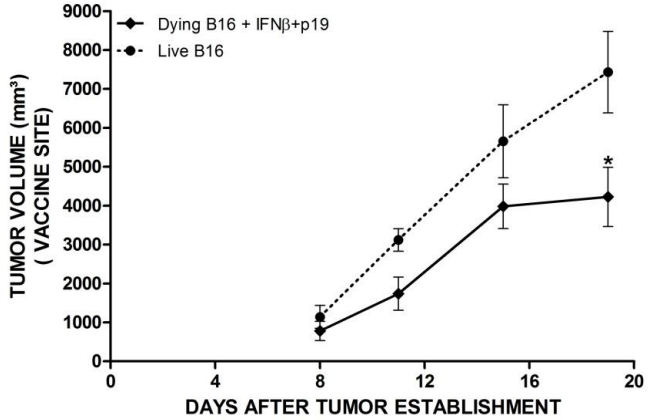
SUPPLEMENTAL INFORMATION OF CHAPTER 4

Supplemental table 1. Primers used for RT-qPCR.

Gene	Primer (5`→3`)	
IL-15	Forward primer	CATTTTGGGCTGTGTCAGTGT
	Reverse primer	AGGCTTTCAATTTTCTCCAGGTC
CCL2	Forward primer	AGCATCCACGTGTTGGCTC
	Reverse primer	CCTCTTGTAGCTCTCCAGCCT
CCL3	Forward primer	TCCCAGCCAGGTGTCATTTTC
	Reverse primer	TCAGGCATTCAGTTCCAGGTC
CCL4	Forward primer	TGTGCAAACCTAACCCCGA
	Reverse primer	CCGGGAGGTGTAAGAGAAACA
CXCL1	Forward primer	CCAAACCGAAGTCATAGCCAC
	Reverse primer	CCGTTACTTGGGGACACCTT
CXCL2	Forward primer	CTCTCAAGGGCGGTCAAAAAG
	Reverse primer	CTCCTCCTTTCCAGGTCAGTT
Raet 1d	Forward primer	CTATGGATACACCAACGGGCT
	Reverse primer	CACTTCATCTGCTGGGGTAGG
Raet 1e	Forward primer	ATGGATACACCAACGGGCTG
	Reverse primer	TCCACTGAGCACTTCACGTC
ULBP1	Forward primer	CATGCCATTGGTGCTCATAGG
	Reverse primer	GGCTTTCCGGTTGTGTTAGTC
H60a	Forward primer	GCACCCACTTCATCCTCTGTT
	Reverse primer	ATGGTTGCTCTTGCTGGTGG
FAS/APO1	Forward primer	CTGCAGACATGCTGTGGATCT
	Reverse primer	TTTCACGAACCCGCCTCC
Killer/DR5	Forward primer	GCGAACTCTGTGCATTCGTC
	Reverse primer	ACCGGAACCAGCAACTTCTT

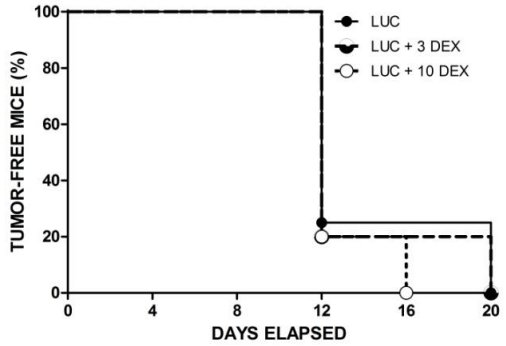
Figures

1



Supplemental Figure 1. Tumor progression in the vaccine site of C57Bl/6 mice vaccinated (3x, once per week) with B16 cells co-transduced with the vectors AdPGp19 and AdPGIFNβ ($3x10^5$ cells) or Live cells (only 1 application with $5x10^5$ cells).

2a



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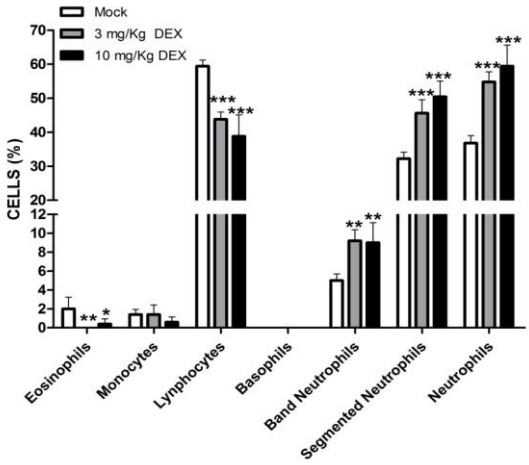

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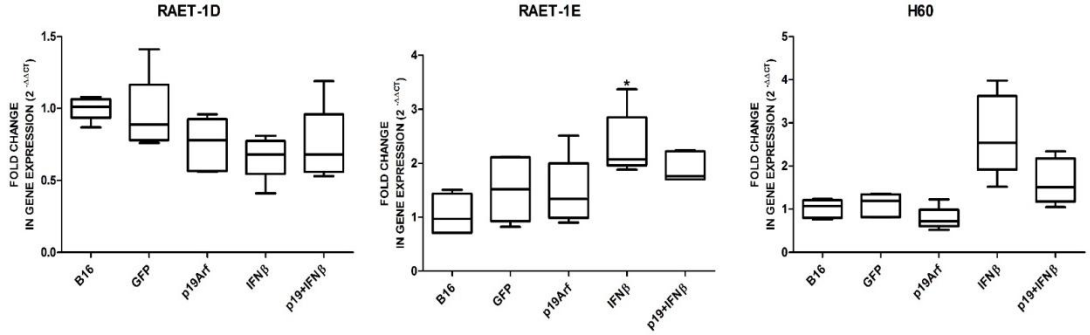
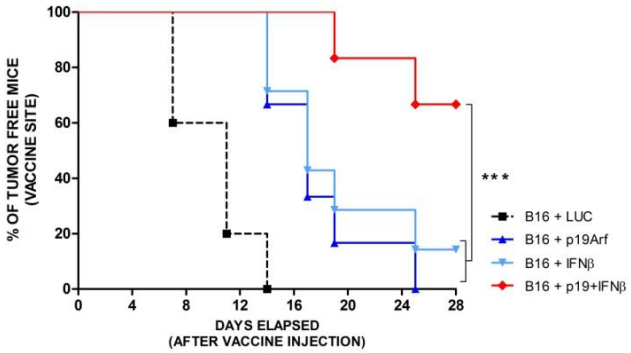
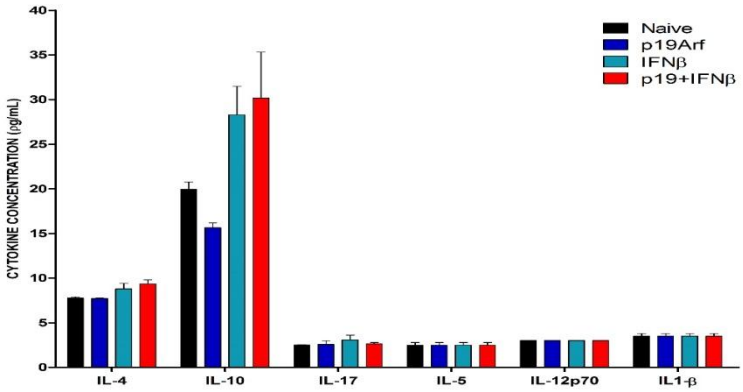


Supplemental Figure 2. **(a)** Tumor onset in the dexamethasone immunosuppressed C57Bl/6 mice. Briefly, immune competent mice were treated for 16 days with 3 or 10 mg/kg dexamethasone and on the eighth day implanted with B16 cells transduced with the AdPGLUC vector ($1x10^5$ cells in a single application). n=5 for all groups. **(b)** White blood cell count. On the twelfth day of immunosuppression treatment, peripheral blood from the LUC, LUC+3DEX and LUC+10DEX groups was collected retro-obritally. [One-way ANOVA followed by Tukey`s Multi comparison post-test]. **(c).** RT-qPCR analysis of gene expression in B16 cells that had been transduced *ex vivo*. Cells were transduced and incubated for 48 hours before purification of total RNA and processing for RT-qPCR. β-actin was used as the reference gene. Data presented as fold change (log2) as compared to the non-transduced B16 condition. Data represent the average and standard deviation from triplicate PCR reactions derived from 3 independent biological experiments. [One-way ANOVA followed by Tukey`s Multi comparison post-test].

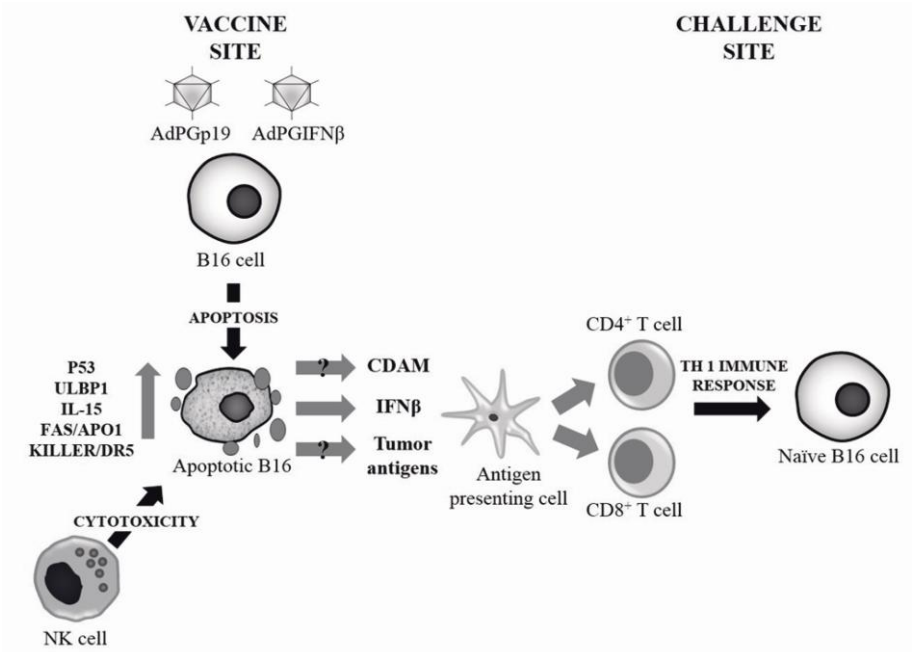
3a



3b



Supplemental Figure 3. **(a)** Tumor onset in the vaccine site. B16F10 cells were transduced *ex vivo* with AdPGLUC, AdPGp19, AdPGIFNβ or the AdPGp19/AdPGIFNβ combination and 48 hours later inoculated (s.c) in the vaccine site of C57Bl / 6 mice. Two vaccinations were made, the first denominated prime with $1x10^5$ cells and the second with $3x10^5$. **(b).** Cytokine expression analysis. B16 cells ($3x10^5$) were transduced as described above and after 48 hours injected into the footpad of C57Bl/6 mice. Ten days later, popliteal lymph nodes cells were collected and co-cultured with fresh B16-LUC cells (stably modified to express luciferase) following the conditions 1:1, 1:10 and 1:20 (B16: popliteal lymph nodes cells) n=4 for the Mock group, n=5 for the $p19^{Arf}$ and p19+IFNβ groups and n=6 for the IFNβ.



Supplemental Figure 4 . Proposed model. Schematic representation of the proposed mechanism of action for the vaccine approach using $p19^{Arf}$ and IFNβ.