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Modelo canino de imunoterapia de melanoma usando vetores adenovirais portadores de cDNAs p14Arf e interferon-beta

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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Canine model of melanoma immunotherapy using adenoviral vectors carrying p14Arf and interferon-beta cDNAs

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RESUMO (ABSTRACT IN PORTUGUESE)

Rodrigues OA. Modelo canino de imunoterapia de melanoma usando vetores adenovirais portadores de cDNAs p14Arf e interferon-beta [tese]. São Paulo: Faculdade de Medicina da Universidade de São Paulo; 2023.

O melanoma é um tipo de câncer agressivo de difícil tratamento em casos avançados tanto na medicina humana quanto na veterinária. Apesar do progresso considerável nas propostas terapêuticas, as taxas de mortalidade ainda são elevadas, tornando necessário o desenvolvimento de novas abordagens terapêuticas. Os melanomas frequentemente apresentam disfunções na via de p53, embora frequentemente retenha a proteína p53 selvagem. Em estudos anteriores, nosso grupo utilizou vetores adenovirais para a transferência genica de p14ARF (proteína supressora de tumor) a fim de reativar a via p53 em células murinas e humanas, tanto in vitro quanto vivo. A co-transdução com IFNβ (citocina imunomoduladora) induziu níveis especialmente elevados de morte celular juntamente com a liberação de marcadores imunogênicos de morte celular in vitro, redução significativa da progressão tumoral e estimulação de fortes respostas imunes in vivo. Antes de prosseguirmos para testes em humanos, desejamos verificar estes resultados em modelo animal que represente melhor a complexidade dos casos humanos de melanoma, incluindo a capacidade de formar tumores espontâneos e metástases. Portanto, este trabalho teve como objetivo desenvolver vetores adenovirais que codificam os genes p14ARF e IFNβ caninos, a fim de validar nossa abordagem em um modelo utilizando linhagens celulares de melanoma oral canino previamente estabelecidas em nosso laboratório. Consistente com nossos estudos anteriores em linhagens celulares humanas e de camundongos, observamos que essa combinação de vetores induziu morte celular acompanhada pela liberação de fatores imunogênicos, como ATP e HMGB1 nas linhagens celulares caninas GAB-F6 e BAN-C10. Num modelo de xenoenxerto de terapia genética in situ, demonstramos a inibição da progressão tumoral in vivo, o atraso no desenvolvimento do tumor e a sobrevida prolongada dos animais. Estes resultados apoiam o futuro teste desta abordagem terapêutica em pacientes veterinários, um passo importante no desenvolvimento da nossa terapia genética para o melanoma.

Palavras-chave: Melanoma, Imunoterapia, Terapia gênica, Interferon-beta, Morte celular, Proteína supressora de tumor p14ARF.

ABSTRACT

Rodrigues OA. Canine model of melanoma immunotherapy using adenoviral vectors carrying p14Arf and interferon-beta cDNAs [thesis]. São Paulo: "Faculty of Medicine, University of São Paulo"; 2023.

Melanoma is an aggressive kind of cancer, difficult to treat in advanced cases in both human and veterinary medicine. Despite the considerable progress in therapeutic proposals, mortality rates are still high, making it necessary to develop new therapeutic approaches. Melanomas often present a dysfunctional p53 pathway, although it frequently retains the wild type protein. In previous studies, our group has used adenoviral vectors for the transfer of p14ARF (tumour suppressor protein) in order to reactivate the p53 pathway in murine and human cells, both in vitro and vivo. Co-transduction with IFNβ (immunomodulatory cytokine) induced especially high levels of cell killing along with the release of immunogenic cell death markers in vitro, significant reduction of tumour progression and stimulation of a strong immune responses in vivo. Before moving to human tests, we wish to verify these outcomes in animal models that more closely represent the complexity of human cases of melanoma, including the capability to form spontaneous tumours and metastases. Therefore, this work aimed to develop adenoviral vectors encoding the canine p14ARF and IFNβ genes in order to validate our approach in a model utilizing canine oral melanoma cell lines previously established in our laboratory. Consistent with our previous studies in mouse and human cell lines, we observed that this combination of vectors induced cell death accompanied by the release of immunogenic factors, such as ATP and HMGB1 in the canine cell lines GAB-F6 and BAN-C10. In a xenograft model of in situ gene therapy, we demonstrated the inhibition of tumour progression in vivo, and delay of tumour development and extended survival. These results support testing this therapeutic approach in veterinary patients, an important step in the development of our melanoma gene therapy.

Keywords: Melanoma, Immunotherapy, Gene therapy, Interferon-beta, Cell death, Tumour suppressor protein p14ARF.

LIST OF ABBREVIATIONS

Ad	Adenovirus
ARF	Alternate Reading Frame
ATP	Adenosine Triphosphate
BAX	BCL2-Associated X protein
BI	Bicistronic
BRAF	Serine/threonine-protein Kinase B-raf
BCS	Bovine Calf Serum
BSA	Bovine Serum Albumin
CAR	Cosackievirus and Adenovirus Receptor
CDKN	Cyclin-Dependent Kinase Inhibitor
cDNA	Complementary DNA
CMV	Citomegalovirus
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
DBP	DNA Binding Proteins
DMEM	Dubelcco's Modified Eagle's Medium
DMSO	DMSO Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double Stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
FDA	Food and Drug Administration

GFP / eGFP	Enhanced Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HDM2	Human Double Minute 2
HEK	Human Embryonic Kidney
HMGB1	Nonhistone Chromatin Protein High-Mobility Group Box 1
ICESP	Instituto do Câncer do Estado de São Paulo
IFN	Interferon
IL	Interleukin
INCA	Instituto Nacional do Câncer
ISGF	Interferon Stimulated Gene Factor
ITR	Inverted Terminal repeat
LUC	Luciferase
LVV	Laboratório de Vetores Virais / Viral Vector Laboratory
MAPK	Mitogen Activated Protein Kinases
MDM2	Murine Double Minute 2
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NK	Natural Killer
N-RAS	neuroblastoma RAS viral (v-ras) oncogene homolog
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde

PI	Propidium Iodide
PTEN	Phosphatase and Tensin Homologue
RIPK3	Receptor Interaction Protein Kinase 3
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TE	Tris-EDTA
TNF	Tumor Necrosis Factor
UV	Ultra Violet

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1. INTRODUCTION

Cancer, the immune system and immunotherapy

All multicellular organisms are a result of cells that have acquired internal programming that prevents their uncontrolled replication, allowing for the orderly development and maintenance of complex structures in the body. In addition, some of the body's cells are specialised in the function of hunting down rogue cells that may disturb this balance. Over time, through intrinsic DNA replication errors or caused by external factors, some cells accumulate mutations that might slowly alter or even turn off their set of instructions. They start to grow uncontrolled becoming a cancer that might stay in situ or, over time, invade nearby tissues. Over time, those cells gain the ability to continue developing and replicating independently of signals from the host body. Moreover, they learn to trespass the bodies defence and hide in plain sight of the immune system. Sometimes even recruiting its cells to support the tumour's development.

In 1909, Paul Ehlich proposed the idea that, contrary to popular belief, the immune system possessed the potential to combat and deter the development of tumours (1, 2). He believed that despite the development of atypical cells during aging, the body had evolved natural ways to suppress them maintaining those cells latent for a period of time. However, without the ability to prove this, his hypothesis was not addressed (2, 3). Years later, Lewis Thomas and Frank Burnet theorised that cancer cell-specific antigens, now known as neoantigens, can induce immune reactions against cancers. By extension, they proposed that there should exist a mechanism for eliminating and inactivating those mutant cells (3). Those ideas plus later discoveries helped to develop what are now known as the theories of immunoediting and tumour immunosurveillance (1)

Tumour immunosurveillance describes a process in which the adaptive and innate immunity components interact with mutant cells to eliminate them, preventing the development of disorders. This first step is called the elimination phase. Due to the genetic instability of some of these cells, some variants that are less susceptible to the immune system's recognition and attack are selected by the evolutionary pressures of this environment. This process of immunoediting leads to an equilibrium phase where nascent tumours do not grow freely nor are they completely eliminated by the natural defences of the body. This phase

can be maintained for many years inducing the selection of a repertoire of cells with the most diverse characteristics. Finally, some tumour cells that manage to survive this selective pressure and acquire the ability to escape the immune system by losing many of the immunogenic antigens they used to express, and also by modulating the activity of immune cells in the vicinity. After a long time adapting to circumvent the immunosurveillance of the body, tumours grow quickly in what is known as the evasion phase (1, 3). In general, most tumours go through these three phases, but in some cases, such as immunodeficiencies and advanced age, tumours can directly enter the equilibrium or evasion phase, without going through elimination.

The elimination phase relies on several mechanisms, including the detection of danger signals such as DAMPS (4, 5) Heat Shock Proteins (HSP) and the release of type I interferons that is induced during early tumour development. These signals activate dendritic cells which lead to a CD4+ and CD8+ T cell response. Another possible mechanism occurs through the expression of Natural Killer (NK) cell ligands, such as NKG2 and TRAIL, leading to the recognition of tumours by NK cells (1). This process, however, is not always successful due to the heterogeneity of the tumours, leading to the survival of variants that have lost the ability to release these signals (1). Later, in the equilibrium phase, adaptive immunity continues working to keep the tumour in a dormant state. In this phase, tumour cells undergo great selective pressure and the development of new variants is increased thanks to the level of genetic instability that accumulates in these cells (1, 5). Over decades, less immunogenic variants are selected and tumours present cell population that suffered loss of tumour antigens, and reduced expression of Major histocompatibility complex class I (MHC I) proteins. Additionally, recent studies have also shown that tumours cells suffer an epigenetic disbalance that leads to non-mutational epigenetic reprograming that helps tumours in this process. Furthermore, some variants acquire the ability to secrete factors that modulate the microenvironment around them, inducing the formation of blood vessels to nourish the tumour, and the modulation of immune cells in the region in favour of the tumour (5). Some organisms induce the recruitment of regulatory T cells (T-reg) which contributes to the creation of an immunosuppressive environment (6, 7). Besides some cells acquire the ability to move through the bloodstream to distant anatomical locations, establishing metastases. These disseminated, secondary tumours will encounter additional selective pressures, leading to the accumulation of unique genetic characteristics. Some of those that will give them resistance to therapies that are effective for the primary tumours (8, 9).

Type I interferons and immunogenic cell death

The interferon (IFN) family of proteins was originally described for their ability to "interferon" influenza virus replication (10). Over time, further studies evaluated the role of these factors against tumours (11, 12). A study has produced mice that are deficient in interferon receptors (IFNRs) or other key pathway factors such as STAT1 that are found to be more susceptible to tumour development than non-altered animals (12).

The cytokines within the IFN family are categorized into three distinct types: type I, type II, and type III. These classifications are based on variations on protein sequence and structure, function, the cells which produce them, and the corresponding cellular receptors engaged by them. Among humans, the type I IFNs encompass 18 distinct members. This includes 13 subtypes of IFN-α, along with single subtypes of IFN-β, IFN-ε, IFN-κ, IFN-τ, and IFN-ω. Intriguingly, all these subtypes bind to a shared cognate receptor, which comprises the Interferon Alpha and Beta Receptor 1 (IFNAR1) and IFNAR2 subunits (13, 14). However, despite sharing the same receptor, each interferon exhibits varied tissue-specific expression (13) , binding affinities, and immunomodulatory outcomes (15, 16). IFN-β exhibits around a 50-fold higher receptor-binding affinity to IFNAR1 compared to IFN-α (17), which results in a more robust antiproliferative response and potentially distinct immunoregulation effects (18).

IFN-α/β, unlike the other type I IFNs, possess more established and well-known roles in immunity (15).

Type II IFNs, has a sole representative, known as IFN-γ. This interferon binds to the IFN-γ receptor 1 (IFNGR1) and IFNGR2 subunits, and is primarily produced by CD4+ helper T lymphocytes and natural killer (NK) cells. Type III IFNs encompass IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4, which interact with the IFN-λ heterodimeric receptor 1 (IFNLR1) and the β subunit of the Interleukin-10 (IL-10) receptor (13, 14)

Activation of the IFN pathway starts with the binding of IFNs to their respective receptors. Each of the receptor subunits (IFNAR1 and IFNAR2 or IFNGR1 and IFNGR2 for IFNγ) interacts with a specific member of the Janus Activated Kinase (JAK) family. Upon IFN binding, JAK proteins come together to form a dimer that undergoes self-phosphorylation, enabling them to trigger the phosphorylation of other proteins in turn. This phosphorylation cascade includes STAT1/2, leading to their association with Interferon Regulatory Factor 9 (IRF9), thus giving rise to the complex referred to as Interferon-Stimulated Gene Factor 3 (ISGF3). This complex plays a pivotal role in inducing the

transcription of genes known as interferon-stimulated genes (ISGs), as illustrated by Figure 1 (14).

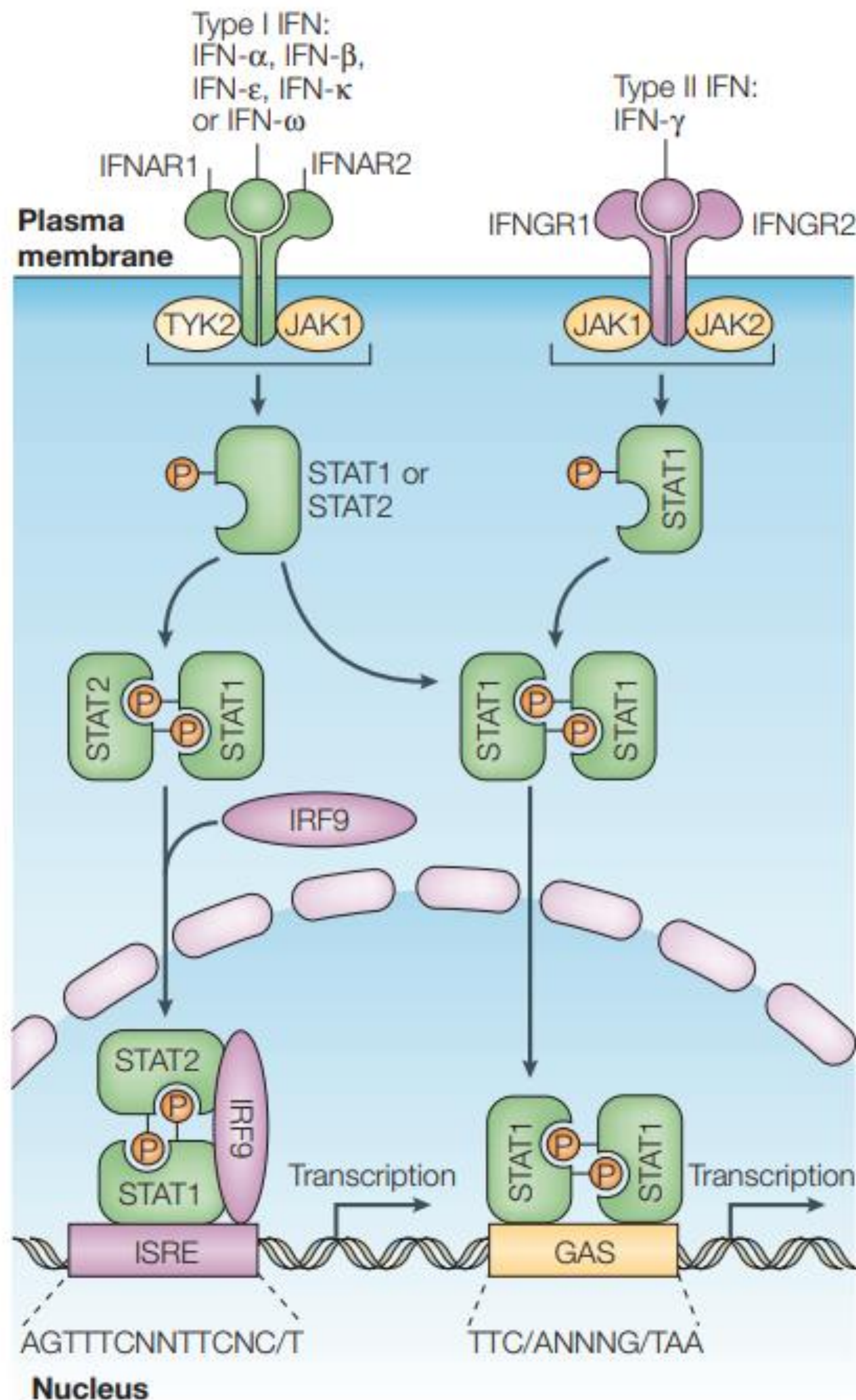


Figure 1 – Classical JAK–STAT pathways induced by type I and type II interferons. Type I interferons and type II interferon stimulates JAK-STAT pathway leading to the transcription of Interferon stimulated genes. Source:(14).

One of the results of activating the interferon pathway is the induction of cell death. All three types of interferons were previously reported to show antitumour capabilities (19, 20). Cancer cells that were exposed to IFNs manifested classic apoptotic traits, including cell shrinkage, membrane blebbing, DNA fragmentation and chromatin condensation (21). However, not all tumours are susceptible to the apoptotic effects of IFNs, which suggests the existence of specific transcriptional patterns that make some tumour cells susceptible to IFN-induced apoptosis, while others are resistant (19).

In the tumour microenvironment context, the delivery or activation of type I interferons induces immunomodulatory and regulatory functions. Usually, a pro-inflammatory environment that favours antigen recognition by tumour associated dendritic cells and the priming of T lymphocytes. Additionally, IFNs type I may increase co-stimulatory molecules of DCs, increasing their capability to cross-present phagocytised tumour antigens to CD8+ T cells (22, 23). Furthermore, they enhance the antigenicity of immunogenic clones by up regulating MHC I molecules (15). On the other hand, it is also known that chronic exposure to type I IFNs can favour tumour progression and immune evasion. The sustained presence of interferons in the tumour environment can stimulate macrophages (mΘ) to produce Interleukin-10, that combined the expression of PD-L1 by immune evasive tumour cells, greatly impairs T cells functions (15). Therefore, despite its possible benefits, therapeutic approaches involving the delivery of interferons must consider this aspect to prevent the induction of pro-tumoral activity.

Also, type I interferons have been also reported by their anti-angiogenic activity. By inducing the reduction of Vascular Endothelial Growth Factor (VEGF) expression IFNs can reduce angiogenesis on the tumour stroma decreasing the flow of blood and nutrients to tumours (15, 24).

Due to their potential role for activating dendritic cells (DCs) (22) recombinant type I IFNs have been approved as immunotherapies for the treatment of cancer (25) such as the recombinant interferon alpha-2β approved by the FDA (23). However, despite the benefits, the systemic use of IFNs can be harmful, triggering side effects, such as fatigue, anorexia, flu symptoms and hepatotoxicity (26). To overcome these complications, DNA transfer methods have been developed (27, 28), concentrating IFNα/β in the area where the vectors are administered, thus the therapeutic effects remain while the side effects of a systemic administration are reduced. Another strategy to reduce side effects can be the combination of IFNs with other immune-stimulating molecules.

Additionally, it has also been noted that the IFN pathway may, under some circumstances, play a role in activating the Rip1/Rip3 complex, also called necrosome (29). Ultimately, sustained necrosome activation leads to the induction of necroptotic cell death in tumour and stroma cells (30-32).

Necroptosis is a programmed form of cell death that resembles necrosis, or inflammatory cell death, due to its potential to trigger the immune system (33). The immunogenic nature of necroptosis favours its participation in certain circumstances, such as antiviral defences (34) or as secondary cell death mechanism that requires apoptosis inhibition. Due to its immune stimulating characteristic, it is considered a type of immunogenic cell death (ICD).

Necroptotic ICD can be stimulated by Toll-like Receptors (TLR3/4/9), the activation of the Tumour Necrosis Factor Receptor (TNFR1) and the Interferon-α/β receptor (29). Despite the existence of alternative routes for the induction of necroptosis, the most described pathway involves signalling via (TNFR1). First, TNFα molecules bind to the TNFR-1 on the plasma membrane inducing the trimerization of TNFRα and release of the SODD domain that otherwise blocks its activation. Then, in this trimerized form, TNFR-1 binds to the TNF receptor-associated death domain (TRADD) and the kinase RIPK1 followed by its association with RIPK3. Then, both kinases phosphorylate each other, preventing the activation of caspase 8. Later, RIPK1/3 dimers associate, forming the necrosome, and phosphorylate the Mixed Lineage Kinase Domain-like protein (MLKL), inducing its oligomerization (Figure 2). The resulting pore-forming structure is translocated to the plasma membrane where it allows the releasing of DAMPs that may then induce the activation of the adaptative immune system (3, 29, 35, 36). TNFα can trigger apoptosis via FADD and Caspase-8, or necroptosis via RIPK3 and MLKL. In cases where caspase 8 is still available, this process ends in apoptotic cell death (36). Many interactions happen between both pathways and it is known that caspase 8 is involved in apoptosis and is able to inhibit RIPK1. Similarly, in some cases, RIPK1 inhibits caspase 8 through the phosphorylation of c-FLIP. The caspase 8 inhibition by c-FLIP as well as an overexpression of RIPK1 favour necroptosis over apoptosis (36).

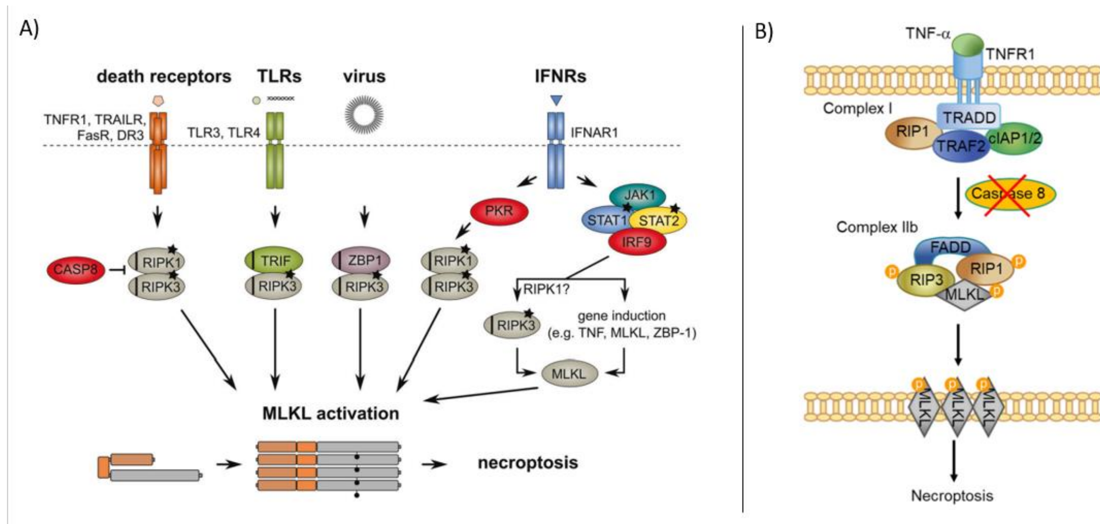


Figure 2 – Necroptosis induction Pathways A) Several pathways lead to necroptotic cell death B) Necroptosis triggered by TNFα pathway. Source: (37, 38).

The induction of necroptosis via interferons is not fully understood, however there is evidence that IFNs can not only induce necroptosis, but also induce transcription of key components of this pathway (39, 40) Experiments have shown that IFNα, IFNβ and IFNγ, in different levels, are able to increase MLKL expression through an intense up-regulation of IRF1 mRNA expression (41). IRF1 is one of the factors encoded by ISGs and also together with the STAT1/2 complex can stimulate other ISGs, among them the MLKL. This phenomenon was observed in HeLa, MV4–11 acute myeloid leukaemia (AML) and MDA-MB-231 breast carcinoma cells (40). Those studies showed no increase in necroptosis due to the simple overexpression of MLKL. However, the up-regulation of MLKL by IFNs in an IRF1- and STAT1-dependent manner induced necroptosis, yet these cells were resistant to apoptosis. Thus, IFN-induced necroptotic cell death may be facilitated particularly in scenarios where caspases are inhibited (40). An additional investigation has suggested that persistent IFN signalling has been identified as a mechanism that sustains a critical MLKL expression threshold, thereby permitting necroptosis (39).

P53 and the immune system

Tumour protein 53 (TP53) is the most often mutated gene in all cancers and its pathway is of critical importance to the cell homeostasis. In normal circumstances, the protein expressed by this gene transcriptionally regulates a network of genes that control many cellular processes (42). The p53 tumour suppressor acts as a central regulatory transcription

factor that becomes active when cells encounter diverse cellular stresses. It oversees numerous fundamental cellular processes, encompassing cell cycle arrest, apoptosis and senescence, DNA repair and the auto-destruction of the cells that are too damaged to be fixed, thus suppressing tumorigenesis (43). Other known functions of this pathways are the regulation of genomic integrity, redox biology, metabolism, non-cell autonomous signalling in tumour suppression and modulation of stemness state (42).

A growing body of evidence, however, suggests that p53's influence extends beyond these roles to encompass immune responses and inflammation (43). Recent investigations have highlighted new transcriptional targets and its potential role in the modulation of innate immunity, NK cells, T-cell response and even immune tolerance (32) and have shown p53 to activate the expression of numerous genes related in the modulation of immune responses (44), including antagonists of immune inhibitors (44). These studies suggest that p53 may be involved in many aspects of immunity through the regulation of key genes involved in the detection of danger signals, antigen presentation, natural killer cells activation, T-cell, induction of interferon release, secretion of extracellular signalling factors, and immune tolerance in cancers (44).

As an example, it was reported that the activation of p53 pathway by DNA damage induces ULBP2 expression on stressed cells. In order to mask their presence, infected and tumour cells often inhibit the expression of MHC and other molecules that can be recognised by the activating receptor NKG2D on NK cells. The ULBP2 protein is one of those molecules (45) and can be alternatively targeted by NK cells. It was reported in two independent studies to be directly induced by p53 (46, 47).

Another well documented gene to be positively regulated by p53 is called TAP1 (48). Its protein in a complex with TAP2 protein mediates the translocation of peptide-antigens from cytosol to the endoplasmic reticulum where they associate with MHC class I molecules to, in turn, be presented to dendritic cells (49).

The promoter region of the ILDR2 gene has a p53 binding site that associates with a mutant p53 (50). This gene belongs to the B7 family, the family of genes that includes PD-L1, known for its role in cancer tolerance through inhibition of T cells (51). Although not yet fully understood, ILDR2 may negatively regulate T cell response, like PD-L1 (52).

Also the p53 pathway was reported to promote the expression of interferon-stimulated genes, including IRF9 transcription factor (53), IRF7, which directly activates the expression of type I interferons (54), and IRF5, involved in pattern recognition by Toll-like receptors (55). In addition, p53, on some occasions, is a transcriptional target of the type I IFN pathway

(44, 56), suggesting a positive feedback loop connecting p53 and IFNs, at least in the context of antiviral responses (43). The link between these two pathways may not be fully understood and future studies may reveal further interactions.

The examples above demonstrate that the p53 plays an important role in the immune system, extending beyond the well-established functions that were originally ascribed to p53. Therefore, the reestablishment of p53 pathway to act as a component in immunotherapy, increasing the efficacy of immune-related approaches by re-establishing immune pathways that were blocked during cancer development due to the inactivation of p53.

Melanoma, incidence and genetics

Melanomas are the most aggressive type of skin cancer, and can appear on the skin, mucosal membranes and uveal tissue (INCA website accessed 27/09/2023, https://www.inca.gov.br/tipos-de-cancer/cancer-de-pele-melanoma). These cancers stem from melanocytes (57), a type of cell originating in the neural crest (58). Among humans, cutaneous melanomas account for the majority of cases, followed by mucosal melanomas with only 0.8–3.7% of occurrences (59).

The Global Cancer Statistics (GLOBOCAN) estimated that, in 2020, non-melanoma skin cancer accounted for 6.2% of all cases of neoplasia and 0.6% of all deaths. Melanoma, despite a 5-fold lower incidence of 1.7%, was responsible for the same death rate of 0.6%, and is the deadliest type of skin cancer, leading to death in 35.3% of all cases (60). In the same year, it was also estimated 325,000 new diagnoses of melanoma. The diagnosis mean age is 65, with 65.7% of diagnoses in people from 55 to 84 (61).

Despite the possibility of effective treatment through surgical removal at early stages, advanced cases of melanoma often lead to the development of metastases and subsequent death. About 10% of melanoma cases are unresectable or diagnosed at the metastatic stage (62), presenting an unfavourable prognosis and a 5-year survival rate around 12% (58, 63). The treatment of advanced melanoma is still a considerable hindrance, as conventional cancer

treatments, such as chemotherapy, are often not effective (64). On the other hand, some targeted therapy and immunotherapy options have improved the general prognosis for many melanoma patients (65).

Melanomas can be classified into cutaneous, acral, mucosal, and ocular (66). Epidemiology, UV-radiation status, histopathological features, genetics, prognosis, and outcomes vary considerably between these subtypes. The most frequent type to affect humans are the cutaneous, followed by acral, mucosal and ocular melanomas (66).

UV radiation is the main etiological factor of cutaneous melanoma. UVA being the most abundant form of sun-light radiation and UVB the more genotoxic one. UVB causes direct DNA damage, while UVA leads to oxidative stress-induced DNA damage (67), often leading to the activation of proto-oncogenes such as BRAF, NRAS, and KIT (32).

Cutaneous melanoma may also be classified as UV-induced and non-UV-induced, according to its risk factors and molecular profile (66). About 75% of deaths caused by skin cancer come from cutaneous melanomas, a percentage that has been increasing in populations of European ancestry (66, 68, 69).

Ultraviolet radiation, especially UV-B, is the main environmental risk factor for the development of cutaneous melanoma (70). It is known that the sun exposure patterns of each individual and the duration of that exposure result in greater or lesser risk.

Mucosal melanoma manifests in the anogenital, oropharynx and paranasal regions, head and neck, parotid glands, oesophagus and middle ear (59, 71) and present frequent local, lung and liver metastases (72). Sun radiation is discarded as a contributing factor because of its location on surfaces not exposed to sun (59). Also, there is no strong evidence of association of this cancer type with human papilloma viruses, human herpes viruses, and polyomavirus infection (73-75). It is suspected that occupational exposure to formaldehyde (76) and cigarette smoking (77) may contribute to the risk of some types of mucosal

melanoma. Even so, the majority of mucosal melanomas are associated with unknown risk factors (59). Furthermore, patients affected by this class of tumour have a 5-year survival rate between 25% and 33% (72, 78).

In genetic terms, melanomas are known to present one of the highest mutation rates of all human cancers (79, 80). Typical somatic genetic alterations detected in melanoma include telomerase reverse transcriptase (TERT), which controls cell replication threshold; cyclin dependent kinase inhibitor 2A (CDKN2A), which encodes genes involved in cell cycle control (81); Histone-lysine N-methyltransferase 2 (KMT2), involved in the regulation of gene expression (82); and Neuroblastoma RAS viral oncogene homolog (NRAS) and BRAF V600E, that encode important factors related to signal transduction (81).Other relevant somatic mutations also occur in the tumour suppressor genes that encode Phosphatase and tensin homolog (PTEN) protein and the TP53 (83, 84).

It is estimated that about 15% of melanomas occur in patients with a family history and often present germline mutation (85). Germline mutations in the CDKN2A gene are linked to the majority of inherited cases and reported to increase the risk of melanoma by 65-fold (32). In those cases, other alterations were also reported in CDK4, TERT, ACD, TERF2IP, POT1, MITF, MC1R, and BAP1 (85). Furthermore, alterations in PTEN, BRCA2, BRCA1, RB1, and TP53, which are related to mixed cancer syndromes, may also increase the risk of melanoma development and progression (85).

CDKN2A encodes two proteins, p16 and p14ARF. Both act as tumour suppressors by regulating the cell cycle. While p16 inhibits cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and activates the retinoblastoma (Rb) family of proteins, blocking the progression of G1 phase to S phase in cell cycle, p14ARF protein (also called p19ARF in the mouse) can activate the p53 tumour suppressor by inhibiting its interaction with HDM2 (86). Besides melanoma, somatic mutations of CDKN2A are commonly found in most human cancers, with

estimates that CDKN2A is the second most frequently inactivated gene in cancer after p53 (86). The loss of p14ARF results in the functional inactivation of the p53 pathway (87, 88)

The p53/p14ARF pathway can be blocked by the overexpression of double minute 2 murine (mdm2 or HDM2 in humans). HDM2 mediates the ubiquitination of p53 protein, leading to its proteasomal degradation (86). As the TP53 gene remains intact in most cases of melanoma, reactivation of the p53 pathway by restoration of p14ARF or inhibition of HDM2 may be viable approaches for fighting melanoma as well as other cancers with a similar genetic profile.

Therapeutic options for melanomas

The main therapeutic approach to combat melanoma is the surgical excision, although radiotherapy and pharmacotherapy are also largely used in combination or when excision is not possible (89). In the clinic, histopathological characteristics of the primary tumour, ulceration status, size, lymph node involvement, and the presence of metastasis are taken into consideration when determining the treatment regimen (90, 91).

Dacarbazine is an alkylating agent approved by the FDA for the treatment of advanced melanoma in 1974. Only 15–25% of human melanomas treated with it show signs of improvement (92). Complete response rates of melanoma patients treated with this drug are less than 5% of cases and survival after 5 years is 2 to 6% (93) In the spite its low efficacy, dacarbazine is still used in the treatment of melanomas as monotherapy or associated with other chemotherapies, targeted therapies or immunotherapy. Apoptosis resistance has been identified among the main causes of melanoma's lack of response to chemotherapy (94). Other agents, such as Temozolomide (TMZ), which is a prodrug derived from dacarbazine, showed a decrease in disease-free progression, although no difference in overall survival was reported (95).

Other drugs used include the platinum derivatives (cisplatin and carboplatin) and mitosis inhibitors (paclitaxel) (96). However, those drugs are not effective in many cases, mainly due to the development of resistance (97-99).

Electrochemotherapy is a low-cost alternative for the treatment of cutaneous and mucosal melanoma. It is a technique that combines the action of cytotoxic drugs such as

bleomycin and cisplatin with high-intensity, short-duration electrical pulses, increasing the cell membrane's permeability, providing a route of entry for the chemotherapy (100). Variations of this approach involving different drugs and also the transfection of plasmids have been investigated (101, 102).

Since 2011, the FDA has approved several therapeutic agents for advanced melanoma. RAF and MEK inhibitors as trametinib and cobimetinib, the selective BRAF inhibitors vemurafenib and drabafenib (57) as well as anti-PD1 and anti-CTLA4 checkpoint inhibitors, such as nivolumab (103), pembrolizumab and ipilimumab (104) were approved for the treatment of cutaneous melanoma, significantly increasing patients survival rate (57). Despite presenting less expression of PD-L1, in comparison to cutaneous melanomas, checkpoint inhibitors also appear to have some level of effectiveness to treat mucosal melanomas (105).

On October 27th, 2015, the FDA approved the first oncolytic virus, talimogene laherparepvec (also called T-Vec) for the treatment of melanoma in patients with inoperable recurrent tumours after initial surgery. T-Vec is a genetically modified vector based on the Herpes Simplex Virus that carries the cDNA for the Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), an activator of the immune response (106). During its clinical trials, a 26% response rate was observed for the treatment of both in situ and metastatic melanoma (90, 107), 16% in a larger group of 436 patients (90) and it was also investigated in combination with other agents (ipilimumab, pembrolizumab and nivolumab) (108) (ClinicalTrials.gov Identifier: NCT02263508).

Despite the increasing number of drugs and recent therapeutic advances, many patients with advanced melanoma are not benefitted, thus motivating continued research and development of alternatives to combat this malady. In addition to novel technologies, the use of laboratory and pre-clinical models that more faithfully represent the clinical condition are needed.

The importance of comparative oncology

Historically, many drugs have presented high attrition rates between development and approval for clinical application (64, 109). In part due to the difficulty and costs associated with the process of drug development (110) but another limiting factor is poor efficacy when those drugs were translated from in vitro and animal preclinical models to human tests (111, 112). Preclinical models often cannot replicate the clinical situation, yet should be chosen so that specific questions may be answered (112).

Traditional preclinical research methods, such as cancer cells that are grown in monolayer or 3-dimensional (3D) cultures, usually cannot simulate physiological drug distribution and interaction with non-targeted cells (64). Murine and murine xenograft models are often the nest step in testing the efficacy of therapeutic drugs intended for human treatment, yet nearly 90% of drugs with proven efficacy in mice fail in human clinical trials (113). Therefore, additional models may be needed.

Comparative oncology is the field of research that investigates the translational relevance of using companion animals to predict the therapeutic effects of new drugs and therapies in humans (64, 114). Many cancers naturally develop in companion animals, such as dogs, cats, and rabbits (64, 115) with high frequency every year. An increasing number of scientific studies are adopting companion animals as models to better evaluate new therapeutic approaches, focusing on the study of specific features that cannot be well analysed only with the traditional models used in research.

Naturally occurring canine cancers offer unique advantages as models for human disease. Spontaneous cancers in pet dogs develop in the presence of an intact immune system, mirroring human conditions (114, 116). Canine cancer exhibits inter-individual and intra-tumoral heterogeneity, metastasis, cancer recurrence, and therapeutic resistance, paralleling human disease characteristics (116). Similar shared environments between pet dogs and humans impact tumour development and progression (117, 118). In addition, they present many similarities to human's genetics (114, 119, 120).

Melanoma is a quite prevalent neoplasia in dogs and more than 4 million new canine cancer cases are estimated per year (117, 121). Upon diagnosis, the usual age of dogs afflicted with either benign or malignant melanocytic tumours is, respectively, 8.1 or 11.6 years (122). Unlike humans, the most frequent type of melanoma within the canine population occurs in the oral cavity (oral/mucosal). The incidence of cutaneous and ocular canine melanomas, paw pads and nail apparatus (acral) being lower (116). The role of ultraviolet (UV) radiation in canine cutaneous melanoma is considered limited, as the animals have a protective coat that diminishes its impact (116). Moreover, the anatomic location of occurrence is closely associated with the biological behaviour of canine melanocytic neoplasia, rendering it a valuable and informative prognostic factor (123). However, despite suspicions that my it be caused by bacterial or viral infections, the exact causes of oral and other UV independent melanomas in dogs are still a riddle (124).

It is widely recognized that human manipulation of canine evolution through generations of artificial selection has led to a reduction in their genetic diversity (114).

Consequently, this process has led to an increase in the accumulation of deleterious genes within canine populations. Interestingly, specific genetic profiles associated with certain cancers in particular dog breeds exhibit remarkable similarities to those observed in human malignancies (114). Germline DNA analysis of dogs with cancer has revealed the presence of genetic alterations that mirror those found in humans. For instance, mutations in well-known human cancer predisposition genes, like BRAF (125), BRCA1 and BRCA2, have been detected in the germline DNA of certain canine cancer cases (32, 126). Common single nucleotide polymorphisms (SNPs) and known copy number variations (CNVs) were also identified (114).

Similarly, alterations in TP53 and related genes, which play a pivotal role in human cancer susceptibility, have also been identified in canine cancer contexts, including melanoma (115, 127). Therefore, canine cancers may be good models for testing drugs that focus on the reestablishment of the p53 pathway directly or indirectly by modulation of up- or downstream regulators.

Furthermore, despite the discrepancies between mutations found in cutaneous and mucosal human melanoma, dog mucosal melanomas present genetic similarities with human cutaneous melanoma. While in humans BRAF is an rare mutation in mucosal melanomas (59), it was reported that canine mucosal melanomas can present cBRAFV595E, which is an orthologous mutation to human *BRAF* V600E (128).

In short, canine melanomas share many similarities with human melanomas, presenting many advantages for its study, specifically as a model for non-UV melanomas (128, 129). Even so, due to some genetic similarities with well stablish targets in human cutaneous melanoma, canine oral melanoma may be useful for testing drugs that target those shared genes and mutations. Additionally, the large availability of dogs as companion animals and the high incidence of cancers in this species favours the use of this model, rather than other large animals that may present similar features but are not as accessible.

Adenoviral vectors

Gene therapy is a therapeutic approach that uses genetic material to treat, prevent or cure a disease or medical disorder. Gene therapy works by introducing genetic material (RNA or DNA) in cells in order to complement a defective gene, restore a missing gene, interfere in the expression of unwanted proteins or even introduce foreign coding sequences that provide

novel functions. This process often occurs by the use of a vectors that facilitate the entrance of genetic material into the cell.

Many types of vectors are commonly used for gene therapy. Some of viral origin and others are non-viral. Each presents distinct attributes that make them more suitable for different goals (Table 1). Therefore, careful consideration should be given to choosing a vector with features that are compatible with the intended application. Some of those characteristics include the vector’s natural or altered tropism; replication capabilities and limitations; immunogenicity; risk of off-target uptake; and insertion of the vector’s genome in the host’s chromosome, which comes with the risk of insertional mutagenesis, but may also ensure long-term expression of the therapeutic sequence.

Vectors	Titre (Viral particles/ml)	Administration route	Expression	Immunogenicity level	Maximum capacity
Retrovirus	10^9	Ex vivo	Long-term	low	8kb
Lentivirus	10^9	Ex vivo	Long-term*	Low	8kb
AAV	10^{12}	In vivo	Transient	Moderate	5kb
Adenovirus	10^{12}	In vivo	Transient	Elevated	9kb

Table 1 – Most commonly used viral vectors platforms and their main characteristic This table represents the most common feature of simple versions of those vectors (130).

Adenovirus is the main viral vector platform used in cancer gene therapy (131), whether as an oncolytic virus or a non-replicative vector. According to The Journal of Gene Medicine Clinical Trial Site (https://a873679.fmphost.com/fmi/webd/GTCT accessed in August 2023), from 1989 to the present, 3790 clinical gene therapy protocols have been approved, with 2622 of these (69.2% of the total) for cancer as a therapeutic target. Regarding type of vectors utilised in those clinical trials, adenoviral vectors still represent the majority of 436 cases (15.0%). Despite the existence of more than 50 serologically distinct types of adenoviruses (132), the human adenovirus type 5 (Ad5) is the basis for the majority of vectors.

Wild type adenoviral particles are non-enveloped and non-integrative particles (130, 133) that present linear, non-segmented, double-stranded DNA genome of about 35-36 kb, approximately 80 nm in size. The adenovirus genome encodes about 35 proteins which are expressed in early and late phases (134)

Adenoviruses natural tropism is for ocular, gastrointestinal, respiratory and urinary tract cells (135). The vectors cell entry mechanism depends on cellular expression of

coxsackievirus and adenovirus receptors (CAR) and αv integrins. In the beginning of the process adenovirus knob attaches to CAR locating the particle next to the cell, then αv integrins connect to the virus fibre base stabilizing their bounding, which leads to clathrin-coated pit formation and subsequent virus endocytosis (136-138) as demonstrated in Figure 3. Additionally, an RGD motif may be to incorporates into the knob protein of adenoviral vectors, allowing transduction of a broader range cells via $\alpha^{v}\beta^{3/5}$ integrins (139-141).

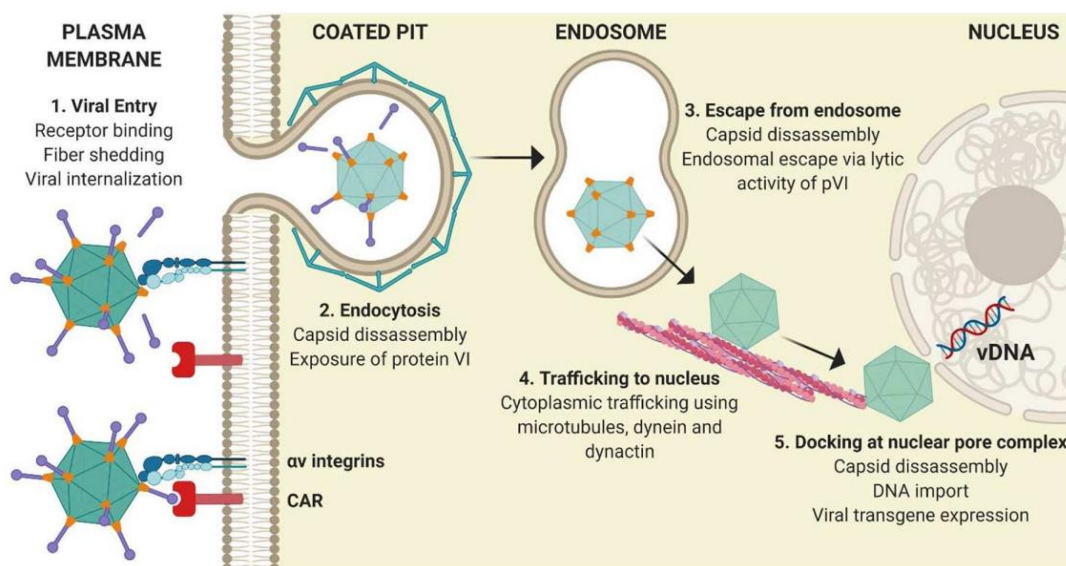


Figure 3 - Adenoviral receptors and classical adenoviral vectors cell entry pathway. (1) Attachment of the Ad5 fibre knob is the first interactions with CAR, which is followed by the interaction of RGD motif within the fibre's penton base with integrins. This is followed by the internalization of virions and (3) endosomal escape. Then the partially disassembled capsid cores traffic to the nucleus and docks on the nuclear pore complex (4). At the end the virus enters the nucleus and inducing transgene expression. Source: (136).

The main issue involving the use of adenoviral vectors is the high frequency of pre-existing neutralising antibodies in the population. These antibodies arise due to prior exposure to adenovirus which is highly immunogenic. In this case, treatment may be ineffective. Even in patients without prior exposure, treatment with adenovirus may result in the rapid production of an anti-viral response, making repeated treatments less effective. In addition, adenovirus particles are efficiently eliminated from the circulation due to their uptake by the liver. Interaction between viral proteins and common features of the liver, such as blood coagulation factors and complement, facilitate hepatic sequestration of the virus and its elimination by Kupffer cells, which in turn direct an anti-adenoviral immune response (142-145). These issues also imply that delivery of adenoviral vectors in the circulation will not be an effective means of reach the tumour.

On the other hand, the immunogenicity of adenoviral vectors may be an advantage for cancer gene therapy. Multiple pathogen-associated molecular patterns (PAMPs) present in the vector may act as adjuvant components to the gene therapy approach (146).

Regarding safety, very few adenovirus subtypes are naturally pathogenic to humans. In the case of vectors for gene therapy use, adenovirus replication capabilities are suppressed and do not replicate within patients' cells. It is achieved by removing the E1A gene from the vector genome (147). During the early phase E1A gene is required to allow the transcription of the followed genes. Also, the proteins encoded by those early-phase genes are essential to start the later phase. Virus DNA replication only occurs at the later phase, about 6 h after infection (148). When the E1 gene is removed from the viral genome, the virus becomes replicative dependent of producing cells expressing E1 gene (147), usually modified HEK293A or HeLa cells. Later genes, transcribed during the late-phase, such as L1, L2, L3, L4 and L5 are responsible for virus assembly and release out of the host cell by cellular lyses (149). Adenoviral vectors may be modified in order to target specific cells, reduce immunogenicity, and avoid interaction with non-target tissues (150, 151). The use of intratumoral administration can also be used to reduce side effects risk by limiting its spread in the body (152).

The reestablishment of p53 pathway and immune induction by p14ARF and IFNβ gene transfer.

As mentioned earlier, TP53 is a prominent gene that acts on the prevention of tumour development. Many tumours are known lose the functionality of the p53 pathway. This occurs by the acquisition of mutations in the TP53 gene, the overexpression of its inhibitor HDM2 or by the loss of the CDKN2A locus, among many other mechanisms.

In order to restore the activity of the p53 pathway in melanoma - a condition recognized for its frequent loss of CDKN2A and maintenance of wild type p53 - the Viral Vector Laboratory at the Instituto do Câncer do Estado de São Paulo (ICESP), focused on the gene transfer of p14ARF (p19Arf in mouse studies) mediated by adenoviral vectors in order to induce cell death. Transfer of the IFNβ gene is expected to stimulate an antitumor immune response. By combining these genes, they may act together to activate p53, induce cell death and promote an antitumor immune response due to the interactions of these pathways. In mouse models, we have shown that p19Arf and IFNβ work together to induce higher levels of cell death than seen by individual gene transfer.

We have developed the AdRGD-PG adenoviral vector which contains modifications that improve tropism and expression of the therapeutic gene. Instead of the native tropism of the serotype 5 adenoviral vector, which depends on the expression of the Coxsackievirus and Adenovirus Receptor (CAR), often lost in cancer cells, RGD tripeptide modification of the virus knob protein directs tropism via integrins. The use of a chimeric, p53-responsive promoter, which we call PG, takes advantage of p53's transcriptional activities. We have shown that the AdRGD-PG vector outperforms standard vectors utilizing the CMV promoter in terms of transduction and transgene expression (152-155).

Use of the AdRGD-PG vector in mouse studies has confirmed the cooperation of p19Arf and IFNβ in the induction of necroptotic cell death and release of ICD markers, activation of NK cells as well as a Th1 response. This approach has been shown effective in vaccination and immunotherapy assays in mouse models of melanoma (156-158).

Studies in human cells, where the AdRGD-PG vector was used to transfer the human p14ARF and IFNβ cDNAs, we have also observed cooperation for cell killing and release of ICD markers (159) (160). Interestingly, we have shown that p14ARF gene transfer is sufficient to induce ICD in a human melanoma cell line (160). We have used an ex vivo model show that gene transfer of p14ARF and IFNβ to a human melanoma cell line can be used to activate dendric cells, T cells and, in turn, the cytolytic capability of these T cells (159). In all, these results suggest that our gene therapy approach may be an effect immunotherapy for melanoma.

Note that species specific differences in cellular response to IFNβ have been reported previously (161). For this reason, we have taken great care to always match the IFNβ cDNA with the cell type in question. Thus, testing our approach in a species other than human or mouse would require the construction of new vectors that encode the cDNAs derived from that species.

As an essential next step in developing our gene therapy approach, we seek to use alternative animal models that more accurately replicate the clinical scenario of human melanomas. The canine model was chosen due to its spontaneous development of tumours; the shared traits between dogs and humans; and similar disease progression and response to therapeutics.

To this end, our laboratory has isolated canine oral melanoma cell lines. In a previous study, we showed that these cells, GAB F6 and BAN C10, are transformed, tumorigenic, harbour wild-type p53, are susceptible to transduction with the AdRGD-PG vectors and can utilize the PG promoter to drive transgene expression (162). With this model of canine oral

melanoma, we will test the AdRGD-PG vectors encoding canine p14ARF and IFNβ for their ability to induce cell death, release ICD markers and inhibit tumour progression in vivo.

2. OBJECTIVES

General goal

The main goal of this work was the production and validation of adenoviral vectors carrying canine p14ARF and IFNβ cDNAs for the immunotherapy of a canine melanoma model.

Specific goals

A. Production of adenoviral vectors encoding the canine p14ARF and IFNβ cDNAs under the control of a p53 responsive promoter (PG).

A.1 Production and purification of AdRGDPG-cARF; AdRGDPG-cIFNβ; AdRGDPG-cARF/cIFNβ;

A.2 Validation of p14ARF and IFNβ expression upon transduction of the canine oral melanoma cell lines GAB-F6 and BAN-C10.

B. Functional characterisation of vectors effects in canine melanoma cell lines.

B.1 Reveal the impact of vector transduction on cellular proliferation: Accumulation of hypodiploid (Sub-G1) cells, MTT, clonogenic assay.

B.2 Evaluation immunogenic cell death markers;

C. Xenograft model (nude mouse) of in situ gene therapy in canine melanoma tumours.

3. METHODS

Cell lines culture

Oral canine melanoma cell lines (CMCL) (GAB F6, BAN C10) were previously established by our group (162). Cell lines were maintained in Dulbecco's modified eagle medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1x Gibco antibiotic-antimycotic (Catalogue 15240062) and kept in humid atmosphere with 5% CO_2 at 37 ° C. The cell lines HEK293A and HT1080 utilised respectively for adenovirus production and an immunofluorescence assay, were also maintained DMEM 10% FBS.

Vector design and construction

A synthetic bicistronic cassette encoding the canine p14ARF and IFNβ genes was designed based on the canine standard DNA sequences from GeneBank [interferon-β (FJ194477.1) and p14Arf (FM883643.1)] and later synthesised by GeneArt GeneSynthesis. Between the genes, a self-cleavable peptide (P2A) sequence was included to split the polyprotein during translation. To facilitate the detection of the canine proteins by western blotting and/or immunofluorescence, tag sequences were included. FLAG (DYKDDDDK) was placed up and downstream of p14ARFThe c-myc tag (EQKLISEEDL) was placed downstream of IFNβ. The inclusion of strategically placed restriction enzyme sites allowed for the insertion of the bicistronic cassette in a pEntr vector that also encoded the p53 responsive PG promoter (pEntr-PG). Inclusion of strategically placed restriction enzyme sites allow for the removal of component as desired from the pEntr-PG constructs, resulting in the pEntrPGcFp14/cIFNbmyc, pEntrPGcp14F/cIFNbmyc, pEntrPGcIFNbmyc, pEntrPGcFp14 or pEntrPGcp14F vectors. Adenoviral constructs were generated by site-specific recombination between the'pEntr vectors and the 'pDest' vector encoding the adenoviral backbone in the presence of Clonase LR (Thermo Fisher Scientific). Afterwards, the desired clones were identified, thus generating the AdRGDPG-FLAG-cARF, AdRGDPG-cARF-FLAG, AdRGDPG-cIFNβ-c-myc, AdRGDPG-cARF-FLAG-P2A-cIFNβ-cMyc-tag (bicistronic) and AdRGDPG-FLAG-cARF-P2A-cIFNβ-cMyc-tag (bicistronic) vectors.

The construction of these adenoviral vectors was initiated by Dr Daniela Zanatta.

Plasmid transfection, virus production and purification

After plasmid expansion in E. Coli and purification, the 10 µg of plasmids were linearised by overnight digestion with *Pac*I, precipitated with ethanol and transfected into HEK293T cells

and after a week, supernatant and cells were collected. This first step of transfection was done in HEK293T lineage, since it is more efficiently transfected than HEK293A. The later steps were performed on HEK293A. HEK293T cells were lysed through three consecutive cycles of freezing in liquid nitrogen freeze, thawing in a water bath (37°C) and subsequently vertexing at maximum speed, during 1 min. Those viral lysates were utilised to infect HEK293A. After observation of cytopathic effect (bubble shape, detachment of 50% of the cells in the plate and formation of clusters of detached cells). The whole process was repeated again always infecting a larger plate. Firstly 35mm, 60mm, 100mm, 150mm until obtaining the production lysates. Those lysates that presented cytopathic effect in 48hs after infection of 150mm plates were aliquoted and stored at -80 ˚C (Figure 4).

In order to produce the vectors, 25 150mm plates were seeded with HEK293A cells and incubated up to 80% confluency. Cells were infected with 25 µL of lysate and incubated for about 48hs. At the harvest point, indicated by the presence of cytopathic effect in 50% of cells, they were collected in 50mL centrifuge tubes, and centrifuged 10 min at 805rcf. The supernatant was discarded, and pellets were re-suspended in 1mL of the medium, and collected in a total of less than 5mL of lysate per vector production. To purify those lysates, each one was submitted to three freezing and thawing cycles to release viral particles. Viruses were added to 2 mL of an iodixanol concentration gradient (m/v) (54%, 40%, 25% and 15%) and the lysate total volume was added into a polystyrene tube (14x89 mm) (Beckman, Cat. N°344059). Afterwards the tube was submitted to one hour of ultra-centrifugation at 35.000 rpm in rotor Sorvall TH- G41 in Beckman Coulter Optima XE-90 Ultracentrifuge. After this process, bands containing complete viral particles were collected with the help of a needle and syringe. To remove impurities of the ultra-centrifugation processes, the total volume was transferred to a Sephadex PD-10 column (GE, Cat. N°52-1308-00 BB). After column purification, glycerol (7% final concentration) was added to the PBS solution containing the virus particles. The viral solution was separated in several aliquots of 10 and 30 µL and stored at -80 °C. The biological titre was determined by using the Adeno-X Rapid titration kit (Clontech, Cat. N°632250), following manufacturer's guidelines.

Canine cells transfection,

Depending on the particular conditions of each experiment, adjustments were made to the cell management protocol, although in general, seeding and transduction OCMCL cells was performed as described below. Firstly, the supernatant of the 10cm plates was collected in conical tubes of 15 ml. Plates were washed with 1xPBS and subsequently trypsinised. After 2 min of incubation in 37°C the cells were collected and centrifuged for 5 min at 200 Relative Centrifugal

Force (RFC). Supernatant was discarded and cells resuspended in 5 to 10 ml DMEM 5% FBS. Cells were counted in a Neubauer chamber the amount of need cell by experiment was seed in a new dish. Afterwards, cells were transduced with 200 MOI of each vector and incubated until the end incubation period of each experiment.

Detection of canine transgenes p14ARF and IFNβ after transduction

In order to detect p14ARF, $3x10^4$ cells per well of HT1080 were seeded on coverslips in 24-well plates and transduced while in suspension with 50 μL of production lysate. Indirect immunofluorescence detection of canine p14ARF (cARF) through Flag-tag peptide was performed. After 24h, cells were fixed in 4% PFA for 10min at 4 °C and then washed with 1X PBS three times, and then permeabilized with 0.5% TritonX-100 in 1X PBS for 10min at room temperature. Blocking was performed with 1X PBS with 5% BSA for 3 hours at 37 °C under agitation. Following blockade, primary antibody [DYKDDDDK Tag Monoclonal Antibody (FG4R) from Invitrogen (Cat. MA1-91878)] was added, diluted in 1X PBS and 1% BSA at 1: 1000 dilution, and processed by incubation for 16h at 4 ° C under agitation. After three washes with 1X PBS for 10min, Invitrogen Alexa fluor 594 secondary antibodies (Cat. A31623) was added (diluted in 1x PBS 1% 1: 1000 dilution) and incubated at room temperature for 1.5h. After 3 washes of 5min with 1X PBS, Hoechst 33342 at 1: 1000 concentration (diluted in 1X PBS, 1% BSA) was added. After incubation for 15 minutes at room temperature with shaking, the coverslips were placed on a drop of 50% glycerol/50% 1x PBS on microscope slides. Images were recorded on EVOS FL Cell Imaging System fluorescence microscope (Thermo Fisher Scientific).

We verified the expression and release of cIFNβ in supernatant through western blotting. For this, 1x10^5 cells per well were seeded in 12-well plates. Cells were transduced in suspension (MOI 200) and then incubated for 48 hours. The culture medium was collected and stored at -80°C. After Tris-glycine polyacrylamide electrophoresis, in a 12% gel, the samples were transferred to a nitrocellulose membrane and incubated anti-MYC-TAG (Novus biolabs NBP2-37822) 1:1000 and subsequently with the secondary anti-mouse peroxidase antibody (Sigma A9044) 1:5000 dilution. Detection was performed through the ImageQuant LASS 4000 using ECL Prime Western Blotting Detection Reagents Amersham (Cytiva, RPN2232).

Evaluation of hypodiploid cells

The impact of the treatments on BAN-C10 and GAB-F6 cell cultures was evaluated by flow cytometry experiments. For this, $1x10^5$ cells were seeded per well in 6-well plates. Cells were transduced while in suspension using MOI 200 per treatment and collected after 72 hours of

incubation. The content of each well was centrifuged (200 RFC for 5 min in centrifuge 5810 r Eppendorf) and the cells were fixed in 70% ethanol at -20 ºC until the time of analysis. Before analysis the cells were washed with 1x PBS, centrifuged and resuspended in 100 µL of a 1x PBS/RNAse solution (50 µg/mL). After 15 min of incubation, 100 µL of propidium iodide solution (5 µg/mL) was added. Then, the cells were analyzed by flow cytometry, under the BL3A filters, on the Attune Life Technologies. Analyses were performed using FlowJo software.

Clonogenic assay

In order to visually verify the potential anti-tumour effects of the vectors and complement the previous assay we attempted a clonogenic assay.
Initially we seeded $1x10^3$ and $1x10^4$ cells per plate in 100mm plates. However due to the distribution of cells and their apparent migration capabilities after 15 days colonies were barely visible in naked eye. Therefore, we performed an adapted clonogenic assay without focusing on the formation of colonies but in the evaluation of the reduction of the covered surface after treatment. For this experiment $1x10^5$ cells (GAB F6 and BAN C10) were transduced (MOI 200) with the AdRGD-PG vectors expressing eGFP (G), cIFNβ (I), cARF (A), both cARF and or the co-transduction of cIFNβ and cARF monocistronic vectors (I+A). They were treated and allowed to grow for 10 days after treatment, fixed and dyed with 0.1% solution of violet crystal.

We intended to analyse and quantify it by imageJ software however the resolution of images was not sufficient to allow the analysis. Therefore, this experiment was used only for qualitative observation of the vectors' impact on the plates.

MTT assay

For this, $4x10^4$ cells were seeded in 500µl of DMEM 5% SFB medium in 24-well plates and transduced with MOI 200 of each vector. After 24, 48 and 72 hours, 50µl of a 5 mg/mL solution of thiazolyl blue tetrazolium bromide (MTT) (Sigma M2128) was added to each well. After 4 hours of incubation, the medium was removed from the wells and replaced by 500µL of DMSO. After homogenization, 100µL/well were transferred to a 96-well plate and the plates were read on the Glomax Explorer plate reader (Promega), at 560nm emission. Absorption values were normalized, and evaluated relative to the control value. The experiment was done 8 biological replicates, and 3 technical ones.

Immunogenic cell death markers

ATP release

For the evaluation of ATP secretion by the cells after treatment with the vectors, 1x10^5 cells were seeded in 12-well plates (500 µL of medium per well), transduced with adenoviral vectors and incubated at 37 ºC, 48 hours, then the total volume of the supernatant was collected and centrifuged to remove possible cells from the medium (200 RFC for 5 minutes at 4 ºC) and then stored at -80 ºC. To measure the level of ATP released in the culture medium in comparison to the control samples, 30 µl of the supernatant and 30 µl of the reagent containing luciferin and luciferase from the ENLITEN ATP Assay System kit (Promega, Cat. No. FF2000) were mixture and the luminescence of the reaction was read on the GloMax Explorer System GM3510 equipment from Promega. The concentration was estimated by comparing the luminescence emitted by the samples with those emitted by standard samples with known concentration.

HMGB1 release

In order to evaluate HMGB1 release, $1x10^5$ cells were seeded in 12- well plates and transduced in suspension with (MOI 200 of each vector). After 48hs of incubation, the medium was collected, the supernatant centrifuged to remove possible cells from the medium (200 RFC for 5 minutes) and then concentrated in Eppendorf concentrator/ vacufuge plus until 20uL and stored at -80°C in. After Tris-glycine polyacrylamide electrophoresis, in a 12% gel, the samples were exposed to a nitrocellulose membrane and incubated with anti-human-HMGB1 antibody (ABCAM ab79823) 1:1000 and subsequently with the anti-rabbit secondary antibody conjugated with peroxidase (Sigma A0545) 1:1000. Detection was performed through the ImageQuant LASS 4000 using ECL Prime Western Blotting Detection Reagents Amersham (Cytiva, RPN2232).

In vivo assays

Balb C nude 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 GAB-F6 or BAN-C10 cells were inoculated in the left flank of the mice, resuspended in 100 µL of 1x PBS. After the establishment of tumours (26 or 38 days respectively), when they developed between 15 and $50mm^3$, mice were treated with four intratumoral injections of 1×10^9 infectious units of AdRGD-PG vectors in a volume of 50 µl per virus, at 48 h intervals. Tumour volume was determined by measurement with a digital calliper. Mice were euthanised before reaching $1000mm^3$, animal weight loss of around 4% of body mass or whose tumours showed signs of

exposed necrosis. Under these conditions, the animals were sacrificed in a CO_2 chamber for 5 min after prior anaesthesia in isoflurane chamber at 4%.

Tumour volume was calculated using the equation below:

Tumor volume = (largest diameter x smallest diameter x smallest diameter) / 2

This project is in accordance with the Law No. 11,794, of October 8, 2008, Decree No. 6,899, of July 15, 2009, and with the rules issued by the Conselho Nacional de Controle de Experimentação Animal (CONCEA), and was approved by the Comissão de Ética no Uso de Animais (CEUA) of the Faculdade de Medicina da USP on 27.06.2018 as protocol number 1088/2018 (Appendix B).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 9 software. The tests used in each experiment are indicated in the legend of each respective figure. P-value lower than 0.05 was considered significant.

4. RESULTS

Production and titration of adenoviral vectors.

The first stage of this work was the expansion of the viral vectors used here, from plasmids previously designed, synthesized and recombined into an adenoviral vector plasmid. These previous stages of construction of the adenoviral plasmids containing canine cDNAs were initiated by Dr. Daniela Zanatta (Viral Vector Laboratory, unpublished data). The canine vectors were transfected into producing cells, expanded, titrated and stored for use.

Briefly, for the construction of the vectors, a cassette encoding the canine p14ARF and IFNβ cDNAs was designed based on the sequences deposited in GeneBank. In order to facilitate the detection of the encoded proteins by Western blot and/or immunofluorescence assay, the cassettes were designed with “tag” sequences (Flag-tag upstream and downstream p14ARF and myc-tag downstream IFNβ). Due to the lack of information in literature about whether up or downstream Flag-tag insertion may disturb the functionality of p14ARF protein, as a precaution, we decided to produce both versions and later select one to continue

the experiments. To enable the simultaneous expression of multiple genes of interest using a single promoter, the self-cleaving peptide sequence (P2A), known to induce ribosomal skipping during translation of a protein, was inserted between the two cDNAs of interest.

Since the synthetic cassette contains several restriction enzyme sites, it was possible to build vectors containing a single cDNA by removing one of the cDNAs from the cassette, as well as any of the additional elements. Cloning was carried out in the entry vector (pEntr) that contains a PG promoter, responsive to p53 (pEntr-PG) and, subsequently, these vectors were recombined with an adenoviral vector (AdRGDDEST), by using the Gateway system from Invitrogen. The AdRGD vector has a tripeptide insertion RGD, previously described by Mizuguchi et al (2001) (163), which gives it broad cell tropism through interaction with heparin and αv integrins. The AdRGDDEST vector was adapted to be compatible with the Gateway system (Invitrogen) (152).

Once the plasmids of the recombinant adenoviral vectors were constructed, we proceeded to the first stage of virus production. Plasmids were linearized and transfected in HEK293T cells. After about a week the content of each plate was collected and lysed as previously described. Each lysate was centrifuged to remove cellular debris and half the volume of the liquid portion was transferred to 35mm plates of HEK293A. At this point, when there was no visible sign that the transfection had been successful, we collected, recovered, lysed, and used the total volume of the lysate for a new infection in same-size plate. This was done up to the maximum of 5 times in order to reduce any risk of vectors genome to recombine with E1 adenovirus gene in HEK293A genome. When the first plate of HEK293A presented cytopathic effect, we moved on the next plate size in order to amplify the number of vectors in the solution. When cytopathic effect was perceived after up to 72 hours, we proceeded to a larger plate until getting a 150mm plate (as shown in Figure 4). Afterwards, we performed assays testing the minimum needed volume of lysate to successfully achieve cytopathic effect in cell in 150mm plates. Then, ee proceed to the expansion in 25 plates of 150mm, purification and titration of each vector. The Ad-RGD-PG-cIFNβ-myc and the vectors encoding Flag-tag downstream p14ARF were expanded, purified and titrated.

In the work described here we utilised AdRGD-PG-cARF-Flag (Ad-ARF); AdRGD-PG-cIFNβ-myc (Ad-IFNβ), AdRGD-PG-cARF-Flag-P2A-cIFNβ-myc (Ad-BI) plus an AdRGD-PG-eGFP (Ad-GFP), AdRGDPG-LACZ (Ad-LACZ) as controls without inserts of therapeutic effect (Figure 5).

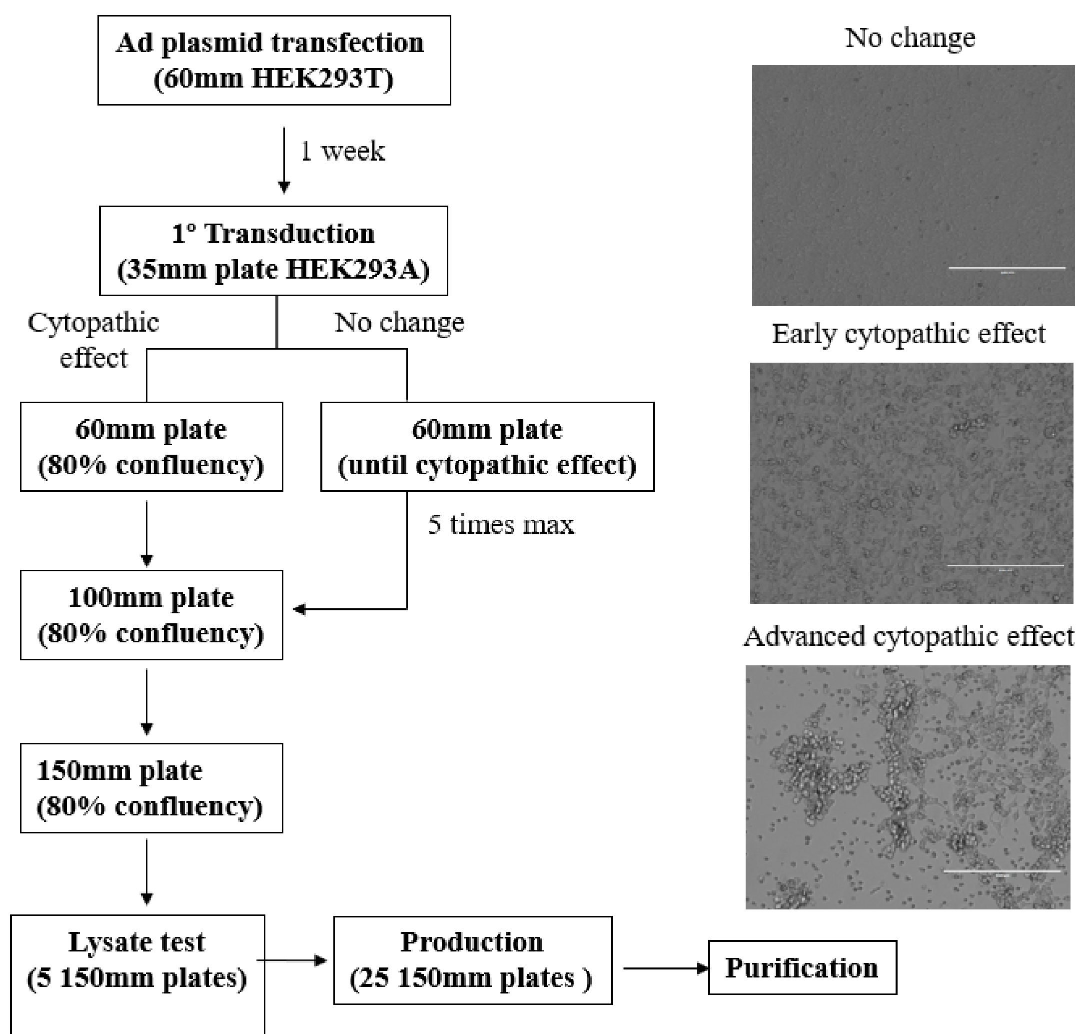
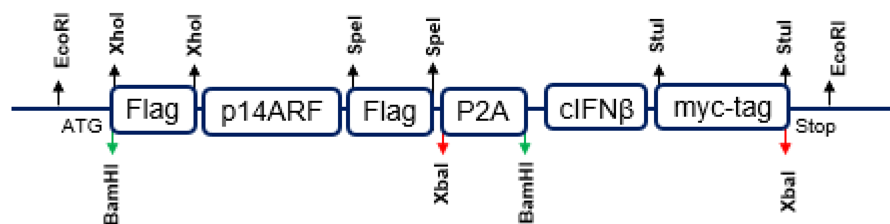
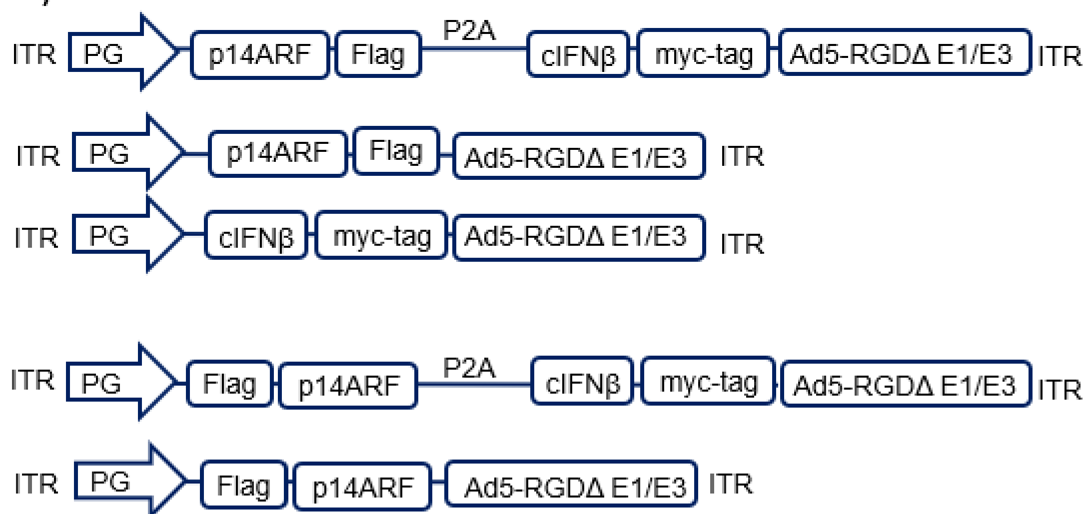


Figure 4: Adenovirus vector production method. Adenovirus plasmids are transfected in HEK293T cells and collected, lysed and used to infect another plate in about a week, when cells present signs of cytopathic effect. If this was not detected after five rounds of infection in plates of the same size, the process was re-started from the plasmid. Once the cytopathic effect has been observed, several steps of lysate expansion are taken until the final production and purification. On the right side, photomicrographs of the expected cytopathic effect. Ideal harvest point is between the early and advanced effect.

A) Transgene cassette



B)



C) Other vectors used as negative controls

Figure 5: Schematic representation of vectors. A) cassette sequence from which all vectors were derived. Black arrows indicate restriction enzyme (RE) sites; green arrows indicate RE sites to remove p14ARF cassette; Red arrows show RE sites to remove cIFNβ cassette. B) Main elements of final adenoviral vectors utilised in this work. C) Control vectors encoding non-therapeutic transgenes. Adenoviral vectors described above are all RGD modified Adenoviral vectors derived from serotype 5, encoding transgenes under the control of the p53 responsive promoter PG.

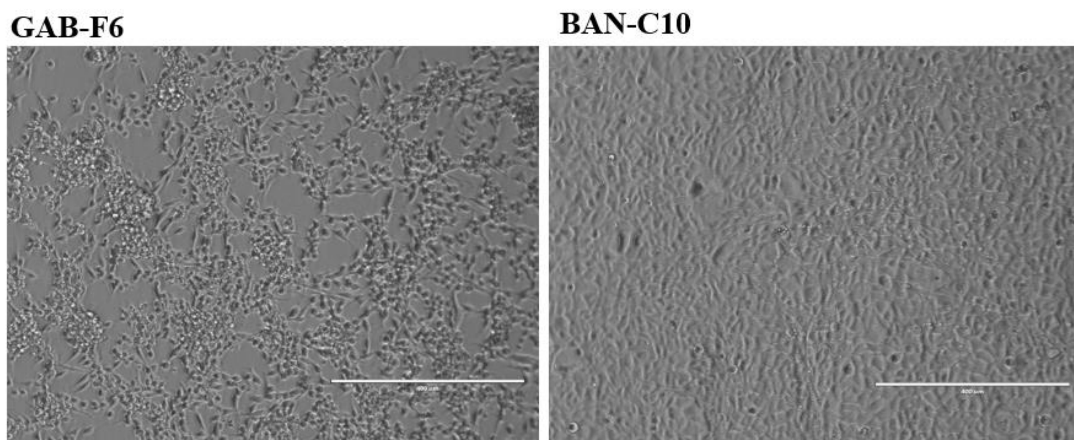


Figure 6: Oral canine cell lines GAB-F6 and BAN-C10

Evaluation of the interaction between adenoviral vectors and canine melanoma cell lines

The oral canine melanoma cell lines (OCMCLs) GAB-F6 and BAN-C10 (Figure 6) were isolated previously in our laboratory and characterized for their susceptibility to transduction using RGD-modified adenoviral vectors (162). To determine the Multiplicity of Infection (MOI, ratio of viable virus particles to number of target cells), we first transduced OCMCL with Ad-RGD-CMV-LacZ, which encodes the beta-galactosidase reporter gene. Cells were transduced with MOI 15, 25, 20, 100 e 200 and fixed, dyed with X-gal and evaluated after 48 hours. Later in that work a similar assay using Ad-RGD-PG-eGFP was performed. Based on those data, it was determined that MOI 200 provided about 85% and 60% transduction of GAB and BAN, respectively, and reliable expression of transgenes while avoiding cellular toxicity.

Validation of transgene expression.

We performed immunofluorescence staining using an anti-Flag antibody to reveal the expression of p14ARF by the newly constructed vectors as well as its subcellular localization in the GAB-F6 and BAN-C10cell line. Firstly, the antibody [DYKDDDDK *Epitope Tag Antibody* (OTI4C5) da *Novus Biologicals* (Cat. nbp1-71705)] presented non-specific staining of the cytoplasm and nuclei of the canine cells. When using the human cell line HT1080, transduced with the same vectors, we detected specific recognition and the expected nucleolar localization of the target protein without any off-target staining (data not shown). Then, in order to confirm p14ARF protein expression and localization in the canine cells, we acquired

an alternative antibody (Invitrogen, Cat. MA1-91878). As desired, this antibody did not present non-specific staining and allowed for the detection of p14ARF, with its characteristic nucleolar localization (Figure 7). We determined the presence of p14ARF in all vectors that carry this gene.

Detection of IFNβ in the transduced cells by immunofluorescence or ELISA was not successful. Instead, detection of cIFNβ-myc protein was performed by western blotting using an anti-myc antibody. This approach was chosen to avoid the detection of endogenous cIFNβ protein expressed by the cells. By using this methodology, we could confirm the expression of the monocistronic cIFNβ-myc encoded by our vector (Figure 7). According to Nishikawa (2000) (164), canine IFNβ protein is known to present at wide band which varies from 20 to 45 kDa, due to its post-translational modifications, thus the protein band detected western blotting is broader than that seen for humans and mice. BAN-C10 presented a less wide band than GAB-F6, possible due to the particularities of the cell lines and the post translational processing of IFNβ. Unfortunately, we were unable to confirm the expression of this gene by the transduction of cells with the bicistronic vector. Further analysis of functional anti-tumour effect of those vectors has also demonstrated that the bicistronic vector was not working properly.

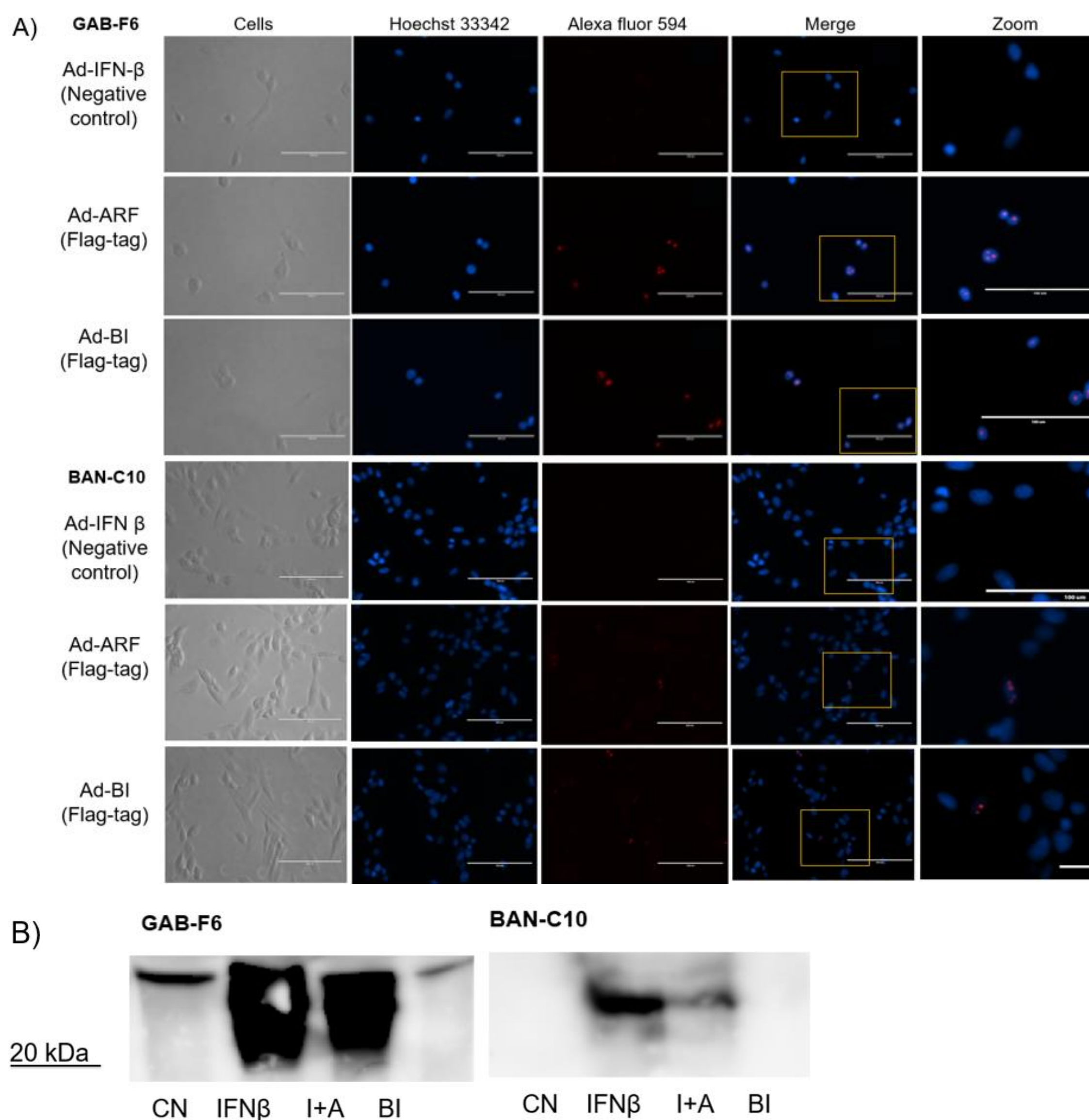


Figure 7 – Transgenes expression. A) Immunofluorescence of GAB-F6 cells transduced with Ad-RGD-PG-cARF-Flag; Ad-RGD-PG-cARF-Flag/cIFNβ; and Ad-RGD-PG-cIFNβ. Red dots mark nucleolar localization of 14ARF-Flag protein. In figure bars equal 100μm. B) Canine Interferon-β detected by western blot. The cell lines GAB-F6 and BAN-C10 were transduced with cIFNβ encoding vectors (MOI 200). Negative controls (NC), AdRGDPG-cIFNβ (I), Ad-RGD-PG-cARF-Flag (A) and Ad-RGD-PG-cARF-Flag/cIFNβ (BI-Flag). Membranes were incubated with anti-MYC-TAG (Novus Biologicals, NBP2-37822) 1:1000 and, Anti-mouse peroxidase (Sigma, A9044) 1:5000. Wide band at 20 to 45 kDa as predicted for cIFNβ due to its extensive post-translational modifications.

<u>Accumulation of cells in the Sub-G1 phase upon transduction with the adenoviral-vectors and clonogenic assay.</u>

We next evaluated the functional impact of the vectors on OCMCL, starting with the accumulation of the Sub-G1 (hypodiploid) population of cells. For this we transduced GAB-F6 and BAN -C10 with the AdRGD-PG vectors expressing eGFP, cIFNβ, cARF, both cARF and cIFNβ (bicistronic vector) or a co-transduction condition of cIFNβ and cARF monocistronic vectors. MOI 200 of virus was used while maintaining equal proportions of transgenes and total viral particles. Therefore, in order to make the total virus quantity and transgene copies more proportional between conditions, monocistronic vector conditions (I and A) were supplemented by the transduction of Ad-RGD-PG-eGFP (ex., MOI 200 A + MOI 200 eGFP, equal to the co-transduction condition of MOI 200 A +MOI 200 I). This way the G+G, I, A and I+A conditions were transduced with same gene dosage and number of viral particles. These results are shown in Figure 8,9.

NC, G, G+G, and BI treatments in GAB-F6 yielded 6.7%, 11.9%, 12.6% and 16% Sub-G1 cells, respectively, with no statistical difference among them. In contrast, 50.3% of cells were observed in sub-G1 for IFN-β and 47.8% for p14ARF ($p < 0.001$ vs. NC). In comparison the combination led to an increase of 67.15% of the population with hypodiploid cells ($p < 0.001$vs. NC). No significant difference was noted between p14ARF and co-transduction conditions.

For the BAN-C10 cell line, 2.4% of sub-G1 cells were observed when treated with the control vector, a lower value than that observed with GAB-F6. Regarding therapeutic vectors, monotherapy led to the accumulation of 51.15% of cells for treatment with IFN-β and 59.9% for p14ARF ($p < 0.001$ vs. NC). When co-transduced the vectors induced the accumulation of 71.7% of the cells in sub-G1 ($p < 0.001$ vs. NC).

Thus, with this test we demonstrated that both in cell lines the control vectors will lead to small amounts of hypodiploid cells, while the combined therapy of IFN-β and p14ARF yielding the highest response rate.

In a parallel experiment, transduced cells were allowed to grow for 10 days after treatment, fixed and stained with a 0.1% solution of violet crystal (Figure 10). With this attempted clonogenic assay, only qualitative assessment is possible since distinct colonies were not observed. At least for BAN-C6, we saw less staining in the presence of the monocistronic vectors. Further optimization is necessary in order to yield quantifiable data. Since the bicistronic vector did not seem to have any effect in either the Sub-G1 or clonogenic assays, possibly due to the lack of IFNβ expression, the remainder of the studies presented here used only the monocistronic vectors.

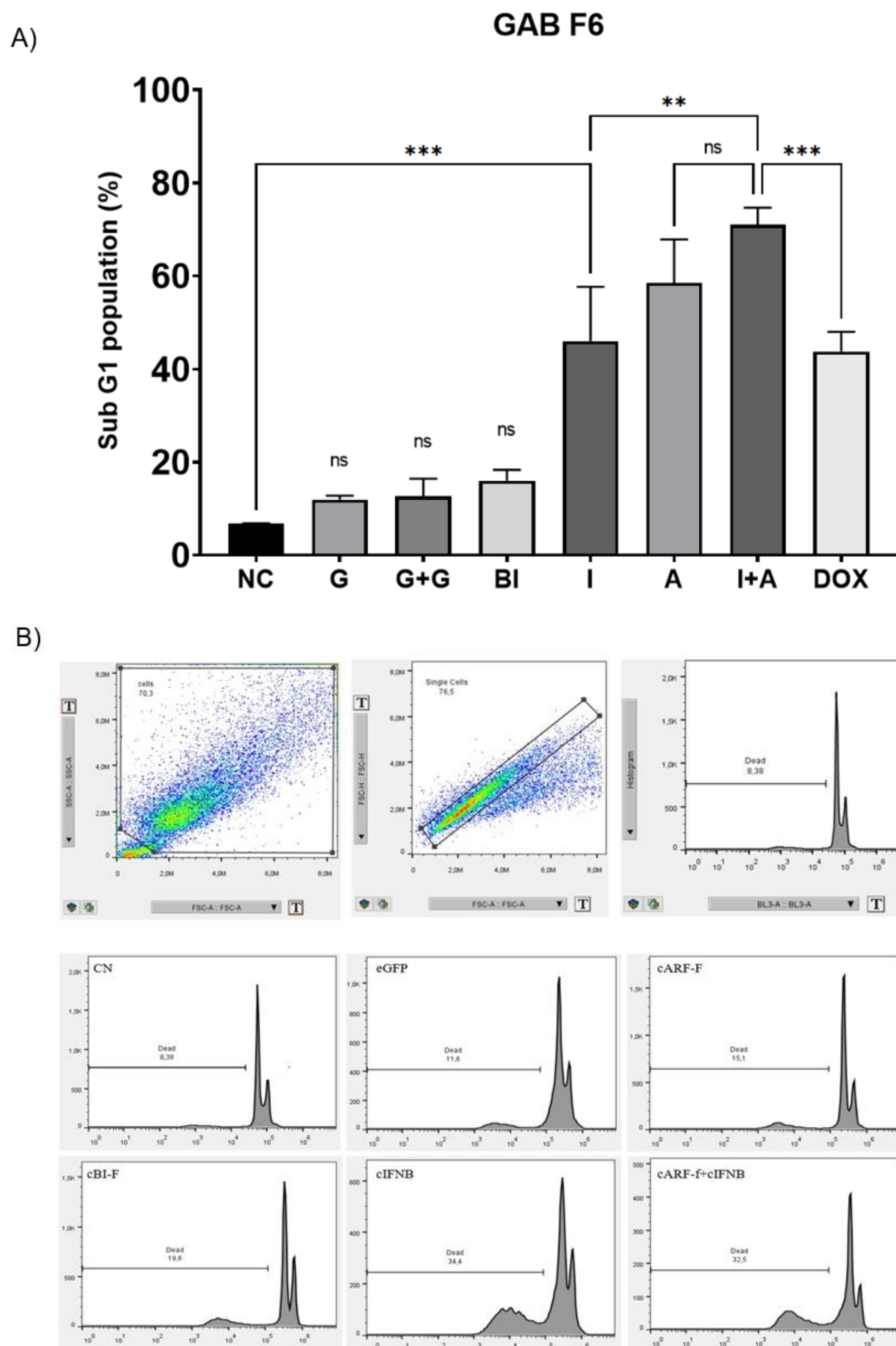


Figure 8 – Evaluation of the GAB-F6 Sub-G1 population by flow cytometry. A) The GAB F6 and BAN C10 cell lines were transduced (MOI 200) with the AdRGD-PG vectors expressing eGFP (G), cIFNβ (I), cARF (A), both cARF and cIFNβ (BI) or the co-transduction of cIFNβ and cARF monocistronic vectors (I+A). Alternatively, 1μM doxorubicin was used as a positive control for the induction of cell death. Cells were collected 72hs after treatment, fixed and stained with PI before flow cytometry evaluation. Data represent the mean and standard deviation between three biological replicates each one with two technical replicates. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$, one-way ANOVA analysis of variance followed by Tukey test. NS, not significant. B) Representative examples of flow cytometry analysis.

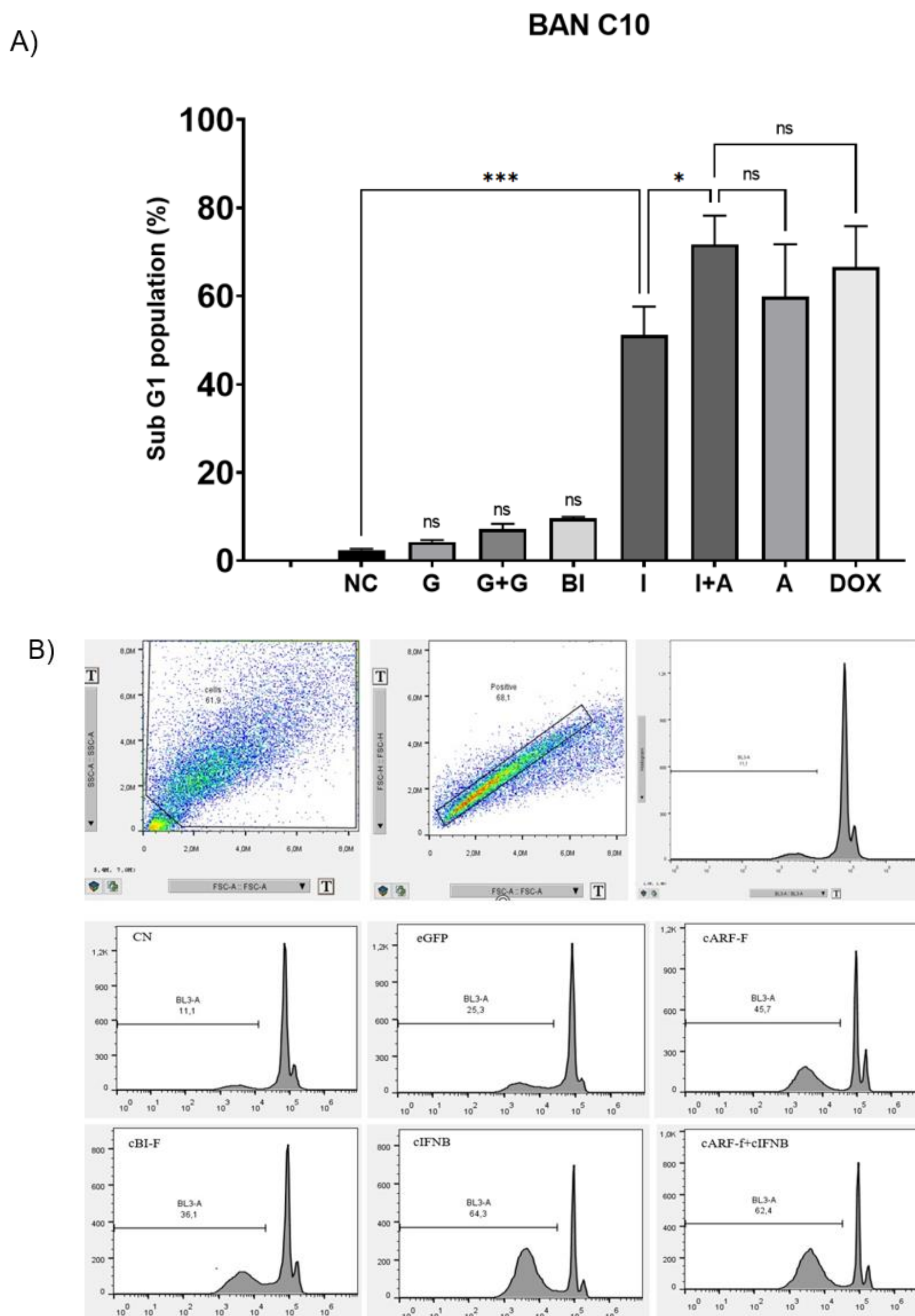


Figure 9 – Evaluation of the BAN-C10 Sub-G1 population by flow cytometry. A) The GAB F6 and BAN C10 cell lines were transduced (MOI 200) with the AdRGD-PG vectors expressing eGFP (G), cIFNβ (I), cARF (A), both cARF and cIFNβ (BI) or the co-transduction of cIFNβ and cARF monocistronic vectors (I+A). Alternatively, 1µM doxorubicin was used as a positive control for the induction of cell death. Cells were collected 72hs after treatment, fixed and stained with PI before flow cytometry evaluation. Data represent the mean and standard deviation between three biological replicates each one with two technical replicates. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$, one-way ANOVA analysis of variance followed by Tukey test. NS, not significant. B) Representative examples of flow cytometry analysis.

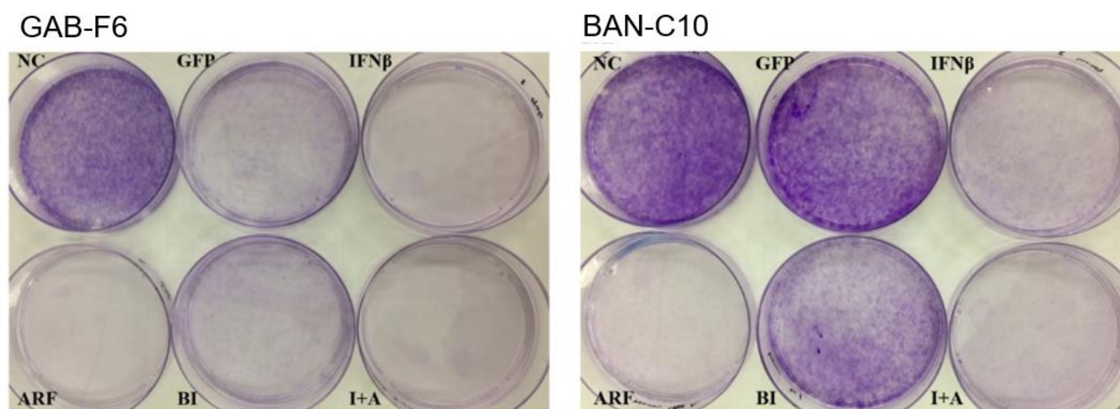


Figure 10 – Cellular response to transduction using a clonogenic assay. For this assay, $1x10^5$ cells (GAB F6 and BAN C10) were transduced (MOI 200) with the AdRGD-PG vectors expressing eGFP (G), cIFNβ (I), cARF (A), both cARF and cIFNβ (BI) or the co-transduction of cIFNβ and cARF monocistronic vectors (I+A). They were allowed to grow for 10 days after treatment, fixed and dyed with 0.1% solution of violet crystal. This assay was performed only once.

MTT Assay

Next, we conducted a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to assess cell metabolic activity as an indicator of cell viability. Figure 11 shows that reduced cell metabolic activity was associated with the transduction with adenoviral vectors in comparison to the negative control condition, especially at later time points. Within 24 hours, the absorbance levels of cells transduced with Ad-cARF and Ad-cIFNβ decreased to 80% and 77% in GAB-F6, and 96% and 90% in BAN-C10. In contrast, the GFP vector controls exhibited minimal change, remaining at 99.2% and 99.7% compared to the negative control.

After 48 hours, GAB-F6 cells subjected to Ad-cIFNβ, Ad-cARF, or the co-transduction of both Ad-cARF and Ad-cIFNβ experienced a considerable decline in metabolic activity levels. Similarly, BAN-C10 displayed a reduction of approximately 50% across all conditions that received therapeutic transgenes. GFP vectors induced some disturbance, dropping absorbances to 87 % in GAB-F6 and 80% in BAN-C10.

Seventy-two hours post-transduction, the relative absorbance levels underwent a decline, reaching a mean of 15.1% in the A+G condition, 14.6% in I+G, and 12.9% in I+A for GAB-F6. BAN C10 exhibited a slightly more moderate decrease, achieving levels of 31% in A+G, 36% in I+G, and an average of 25% in I+A. Despite the substantial difference of

these levels of absorbance to the negative control and the GFP reporter vector condition, the other three treatments did not display significant variation among themselves.

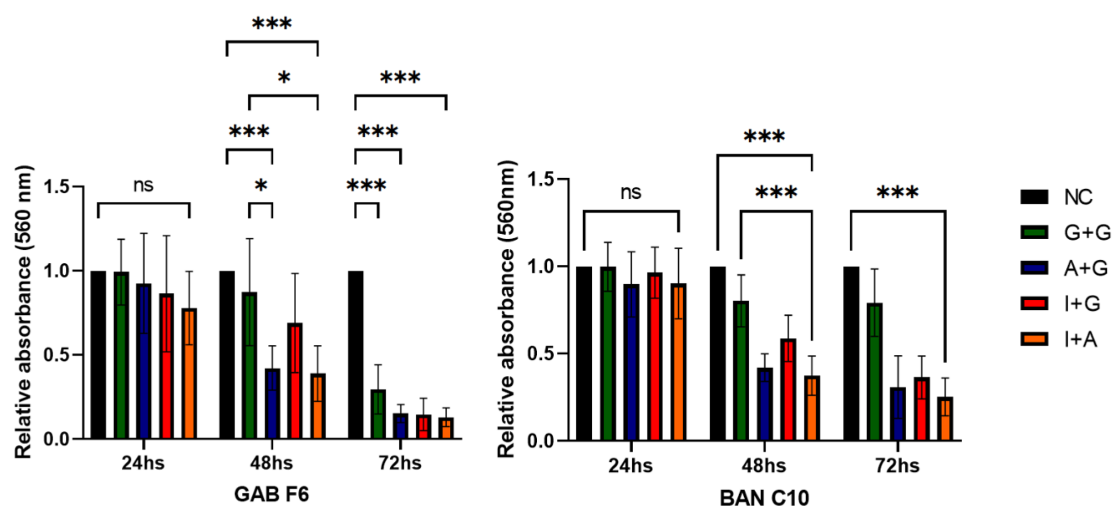


Figure 11 – Metabolic activity (viability) detected in an MTT assay. OCMCL GAB-F6 and BAN-C10 were transduced (MOI 200) with AdRGD-PG vectors expressing eGFP (G), cIFNβ (I), cARF (A), co-transduction of monocistronic vectors encoding cARF, cIFNβ (I+A) and non-transduced cells (NC). Data represent the mean and standard deviation of 8 biological replicates, each with 3 technical replicates. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$, two-way ANOVA analysis of variance followed by Tukey's test. NS or absence of the comparison indication means not significant.

<u>Release of immunogenic cell death markers by transduced cells</u>

We then investigated the release of immunogenic cell death markers upon transduction of the melanoma cell lines. The results show that treatment with the adenoviral vector particles alone was not enough to induce significant cellular stress in either cell line. In GAB-F6 cells (Figure 12A), transduction with the p14ARF encoding vector alone, the detection of ATP in culture medium was about 40 times greater than in negative control and GFP conditions. The mean level registered for co-transduced cells was about 80-fold higher vs. GFP. Oddly, BAN-C10 (Figure 12B) did not result in significant alterations in ATP release upon transduction.

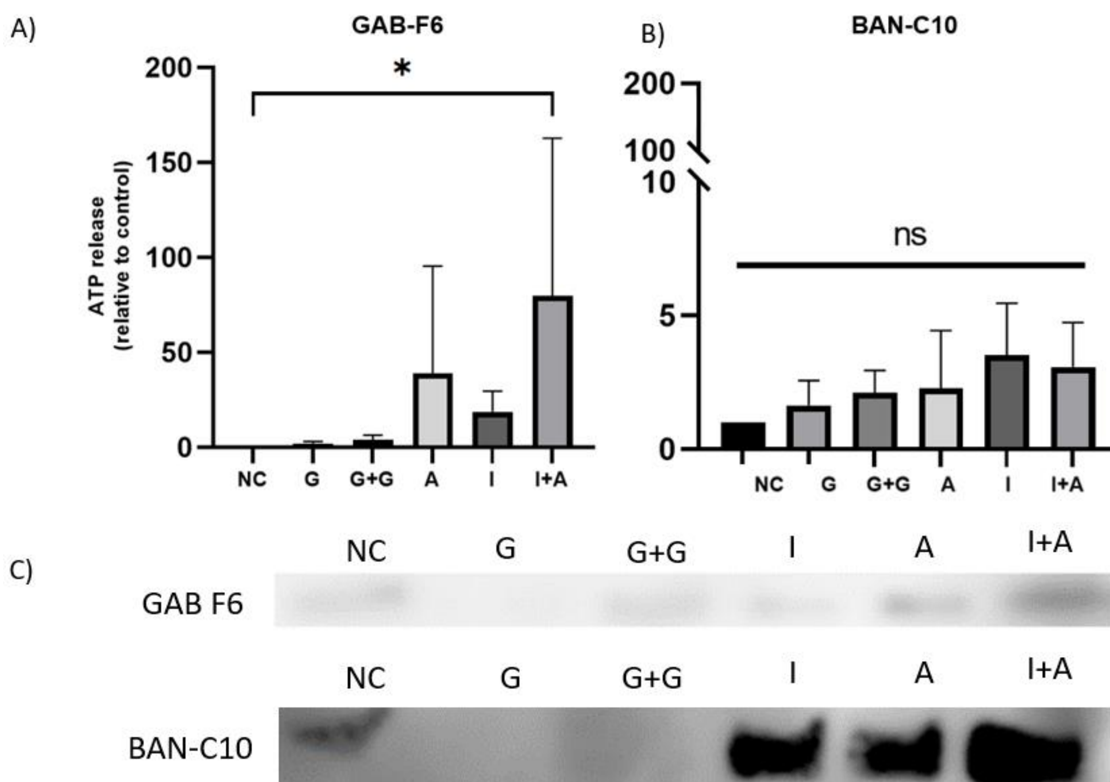


Figure 12 – Immunogenic cell death markers. A, B) ATP release upon treatment. Cells were transduced as indicated and cell supernatants were collected 48 hours later, mixed with Promega ENLITEN™ ATP Assay System reagent in equal proportions and analyzed at integration time of 0.3s. Data represent the mean and SD between 4 normalized biological assays, each with two technical replicates. * P<0.05; ** P<0.01 and *** P<0.001, non-parametric Kruskal-Wallis followed by Dunn. NS, not significant. C) HMGB1 detection. For detection of HMGB1 released in the culture medium. Supernatant was mixed with anti-HMGB1 antibody after 48 h of incubation at 1:1000 dilution and secondary antibody at 1:1000 (BAN) and 1:5000 (GAB) dilution. NC stands for negative control (no treatment); G single and G+G double amount equalising the co-transduction condition (Ad-GFP); I (Ad-IFNβ); A (Ad-ARF); I+A (co-transduction of Ad-ARF and Ad-IFNβ).

We next observed that both cell lines exhibited some level of HMGB1 release in the cell culture supernatant upon treatment (Figure 12C). In BAN-C10, the release of HMGB1 is present in IFNβ and p14ARF transduced cells and also in co-transduced cells, but not in control conditions. In GAB-F6, the presence of HMGB1 was only visible in p14ARF and co-transduction. Altering the dilution of the secondary antibody yielded an improved image for BAN-C10, but has not yet been attempted for GAB-F6.

Evaluation of the intratumoral treatment with the vectors in in vivo xenograft mouse model

In an effort to validate the potential of our approach to inhibit tumour growth, we used a xenograft mouse model of in situ gene therapy. For this, we implanted $1x10^6$ GAB-F6 and BAN-C10 cells in BALB C nude mice. Once the tumours reached approximately 60 mm^3, we initiated treatments. Four intratumoral inoculations of $1x10^9$ viable virus particles were given to each mouse, at 48-hour intervals. Animals received PBS or a virus carrying eGFP as controls, or the experimental conditions Ad-ARF, Ad-IFNB, and co-transduction.

For the GAB-F6 cell line (Figure 13), it was observed that animals belonging to the groups receiving IFN-β alone or in combination with p14ARF displayed a comparable inhibition of tumour progression and survival. Mean size of tumours 190.54 mm3 (IFN-β alone) 173.08 mm^3 (Co-transduction) in day 16, and in contrast, NC tumours size mean was of 527.86 mm^3. However, the p14ARF treatment alone exhibited less efficacy compared to the other two therapeutic transgene conditions (mean of 313.17 mm^3). Similar patterns were observed for tumours originating from the BAN-C10 cell line (Mean size of 432.03 mm^3 for Ad-GFP, 364.64 mm^3 NC, 332.2 mm^3 Ad-ARF, 57.58 mm^3 Ad-IFNβ, and 34.66 mm^3 co-transduction in day 40) (Figure 14).

The Kaplan-Meier survival curve for animals inoculated with GAB-F6 cells exhibited enhanced survival time for those subjected to IFN-β and the co-transduction condition, with one animal remaining alive at the conclusion of the follow-up period (Figure 15). Regarding the BAN-C10 groups, both the IFN-β and combination conditions extended the duration of animal survival (Figure 15). Nevertheless, on day 108 of the follow-up, mice from all therapeutic conditions had reached the permitted ethical threshold for tumour growth and were euthanised.

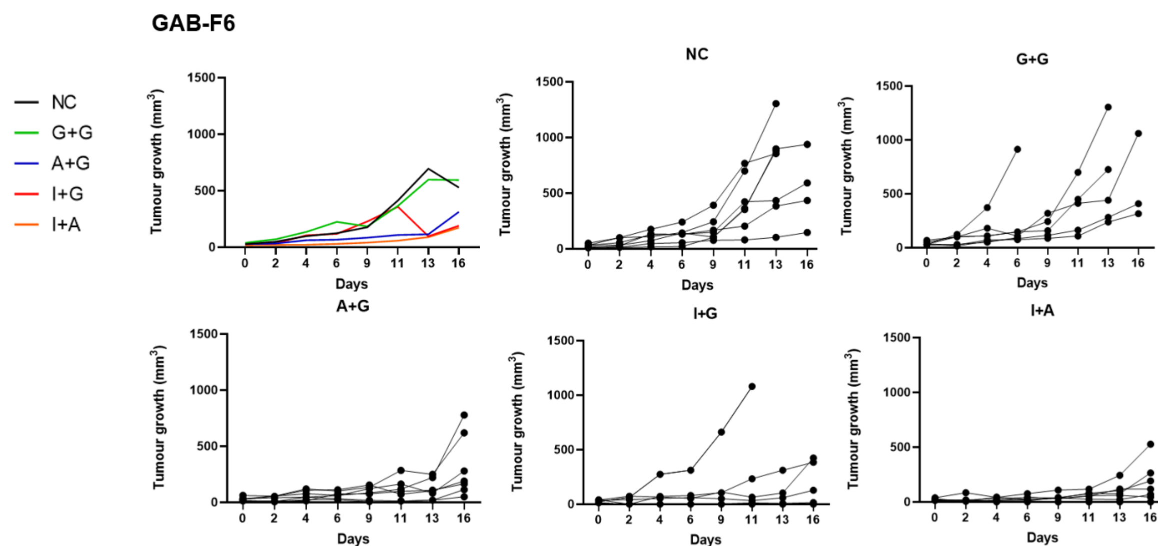


Figure 13 – GAB-F6 in vivo tumour growth. Tumour growth curve of mice inoculated with GAB-F6, treated with adenoviral vectors (days 0, 2, 4 and 6). Animals were treated with adenoviral vectors and followed up to assess the initial impact of treatment on tumour progression. /). * P<0.05; ** P<0.01 and *** P<0.001, One way ANOVA followed by Tukey.

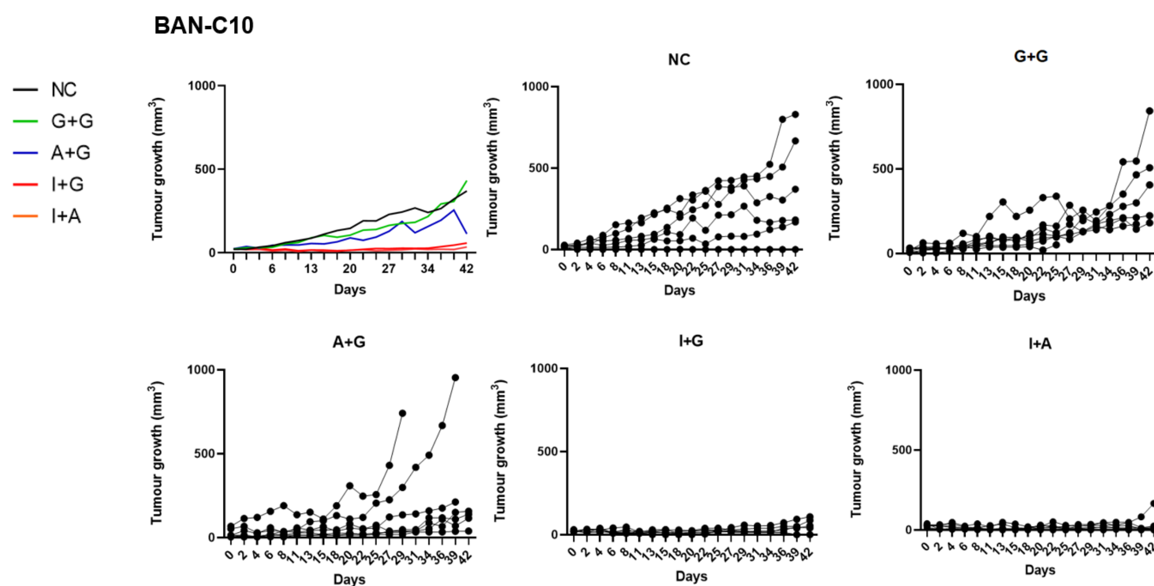


Figure 14 – BAN-C10 in vivo tumour growth. Tumour growth curve of mice inoculated with BAN-C10, treated with adenoviral vectors (days 0, 2, 4, and 6). Animals were treated with adenoviral vectors and followed up to assess the initial impact of treatment on tumour progression. /). * P<0.05; ** P<0.01 and *** P<0.001, One way ANOVA followed by Tukey.

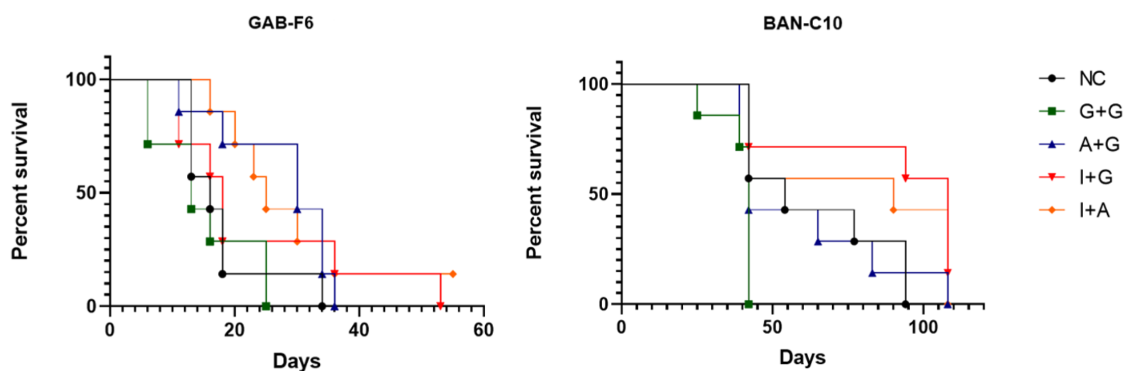


Figure 15 – GAB-F6 and BAN-C10 overall survival. Kaplan-Meyer survival evaluation of animals until the period of treatment until euthanasia according to the ethical limits. (Survival absence of necrosis or loss of weight and tumour size <1000mm^3). NC – PBS 1X, G - eGFP vector, A - p14ARF vector, I – IFNβ vector, I+A – co-transduction. Treatment conditions G+G, A+G and I+G received eGFP vector complementation to adjust the dose of total viral particle and DNA in relation to the I+A condition. Data from A and B, represent mean and standard deviation (N = 7). P value = 0.0037 Mantel-Cox test.

5. DISCUSSION

Vectors production and functional validation

The work presented here elucidated some molecular and cellular features induced by adenovirus-mediated gene transfer of p14ARF and IFNβ to oral canine melanoma cells.

Initially, we ordered the construction of a synthetic cassette containing the cDNA sequences of canine p14ARF and IFNβ. Subsequently, this cassette was used to generate five distinct arrangements of the transgenes, all of which were recombined into a plasmid encoding a previously modified adenoviral type 5 genome. In this modified configuration, acquired for previous studies of the group, is an engineered Ad with tropism for a broader spectrum of cell types. Adenovirus serotype 5-derived vectors rely on the presence of the Coxsackie Adenovirus Receptor (CAR) on target cells for successful transduction. In our research, we employed the use of vectors that have previously undergone modifications to overcome this issue. These vectors have been enhanced by the incorporation of the tripeptide RGD (arginine-glycine-aspartic acid) in its fibre knob. This permits the vectors to transduce a broad spectrum of cells expressing integrins, even in the absence of CAR. However, the level of integrin also directly correlates to the level of transduction of each target cell (165, 166).

Another key alteration in those vectors, in comparison to the most classical adenoviral vectors type, involves the incorporation of the p53 responsive promoter, namely PGTxβ or

PG (155). It is worth mentioning that this promoter exhibits a modest baseline expression that becomes significantly augmented upon the restoration of the p53 pathway, thereby intensifying its expression levels. It was noted that this promoter exhibited up to 5-fold greater strength compared to the CMV promoter, when induced upon the presence of functional p53 (155).

After the completion of plasmids encoding the adenoviral vectors, they were transfected into HEK293T cells for the production of viral particles. Subsequently cell lysates were amplified in HEK293A cells in order to increase the number of viral particles per volume of lysate. This amplification step is already known to present some hindrances, especially during the expansion of transgene expression vectors with therapeutic potential. Some transgenes known to have antiviral or cytotoxicity activity have been reported to interfere with virus replication or sometimes to induce death pathways in the producing cells, interrupting viral synthesis. For instance, vectors carrying Tumour necrosis factor (TNF) (167) and Fas ligand (FasL); (168) are reported to exhibit cytotoxicity that results in the reduction of virus production.

During the course of this work, we encountered some challenges in the process of expanding lysates containing IFNβ. This outcome is understandable, given its recognized antiviral activity (169, 170). Furthermore, in some of the transfection attempts, we believe that the transfection was unsuccessful. However, the evaluation of the transfection depends on a visual observation of the cytopathic effect that is not always present in the initial stages of expansion. The cytopathic effect is represented by the visualization, under a microscope, of 50% of detached cells with the possible formation of cell clusters. In addition, another observation that indicates the cytopathic effect in cases of difficult visualisation is the morphology of the still adhered cells that are swollen due to the production of viral particles that have not yet been released.

In the initial stages of expansion, due to the transfer of lysates from one plate to the next, cellular factors can often contribute to some level of induction of cell death, leading to difficulty in identifying the cytopathic effect. After many attempts at transfection and a few repetitions of amplification in 60mm plates, we were able to expand the lysates to 150mm plates and subsequently carry out virus productions on a laboratory scale for use in the experiments (As demonstrated by Figure 4). During this step, we performed some previous assays with lysates to verify gene expression. However, carrying out further tests with a defined amount of virus was only possible after the production, purification and quantification of all vectors.

With the initial obstacle overcome, tests were initiated to determine the susceptibility of oral canine melanoma cell lines to transduction via adenoviral vectors. With these experiments, in which we used LacZ and GFP encoding vectors, we ascertained to the best multiplicity of infection for the next experiments (162). In other words, we determine how many viral particles should be applied on canine cells to allow the transduction of the majority of them. Once MOI 200 was standardized for the next experiments we proceeded with the functional assays.

We performed a validation test in order to verify the whether the transgenes encoded by the vectors were expressed in canine cells. With this we aimed to confirm the cassette design of the vectors is functional and able to correctly express its genes. We initially faced some hindrances when trying to visualise p14ARF by immunofluorescence using an anti-Flag antibody. However, despite our precautions, we verified an immunofluorescence pattern that was spread throughout the entirety of the cells. Considering the contrasting observations that did not corresponded to the literature, we performed this experiment in HT1080 human cell lines. As suspected, the antibody presented unspecific labelling in canine cells, which was not observed in human cells. Based on these results we purchased another antibody, successfully visualising the p14ARF-Flag or Flag-p14ARF transgenes in the nucleolus of canine cells, as predicted by the literature (Figure 7A). As we firstly develop a full set of vectors with Flag-tag downstream p14ARF, we started the functional assays using those vectors along with the IFNβ vector.

Afterwards, we performed a western blotting assay to verify the expression and liberation of IFNβ by transduced cells. We successfully verified the accumulation of IFNβ in cell culture medium. In the Figure 7B, both Ad-IFNβ and co-transduction conditions show this protein. When co-transduced, the release of IFNβ is slightly lower than in mono-vector transduction. Which may be related to a lower expression due to viral genomes competition for cellular resources. The bicistronic vector, however was unable to express IFNβ protein. With this, we suspect that the P2A element in the vector is not functioning as expected, not allowing the separation of the proteins. Alternatively, ribosomes may be disconnecting from the mRNA before translating the cIFNβ gene sequence. It is also possible that p14ARF and IFNβ undergo subcellular processing by different mechanisms that are not supported by the bicistronic arrangement. We cannot rule out errors in the sequence of the synthetic cassette, but this seems unlikely since the monocistronic IFNβ vector was reliable.

In vitro determination of fractional DNA content and indications of reduction of cell viability

Subsequently, we proceeded with the evaluation of the induction of accumulation of sub-g1 cells in flow cytometry, which is an indicator of cell death. In Figure 8,9 we observed that all vectors encoding some a therapeutic transgene, with exception of the bicistronic vector, were able to induce high levels of sub-g1 events. Results that may suggest their direct cytotoxicity upon transduction. It was also noted that GAB-F6 culture presents a relative natural increased sub-g1 population on flow cytometry. We tried to determine whether it was related to manipulation of the cells or the passages of the culture. Even by cultivating the cells from the original stock, stored from the time of isolation of the culture from dog melanomas, the same pattern was observed. This indicates that the observed level of sub-g1 cells in the absence of treatment probably is an intrinsic trait of the GAB-F6 cell line.

In a parallel with the above experiment, we attempted to perform a colony assay by transducing equally amounts of cells, which were let to grown for 10 days after treatment (Figure 10). This experiment successfully demonstrated the impact of the vectors on cell growth. Nonetheless, we could not count colonies as originally planned. Both canine cells lines used in this work tend to spread and do not form well defined colonies, making visualization difficult, even upon crystal violet coloration. Therefore, despite the lack of quantification we decided to show this result as a qualitative illustration of the effect of those vectors on the replication of OCMCL.

Furthermore, considering the absence of functional outcomes from the bicistronic vector as evident in the results depicted in Figures 5B, 6A, and 7, we decided to exclude the bicistronic vector from subsequent assays. Based on these results we also hypothesised the possibility of the non-split of the encoded proteins during translation, what might have allowed the expression of a chimeric protein. These hypothetical polypeptides could allow the detection of p14ARF on the immunofluorescence assay without permitting its functional performance on other tests. Otherwise, whether the P2A was not allowing the translation of the second transgene the p14ARF should not be ineffective. Thus, allowing similar performance as monocistronic transduction of p14ARF encoding vector, in all other tests.

Later, to assess the short-term cytotoxic effects of the treatments, we conducted an MTT colorimetric assay, examining the impacts of treatments at 24-, 48-, and 72-hours post-transduction. Figure 11 depicts an initial decrease in cell metabolic activity the was superior in the co-transduction condition. Moreover, after 72 hours, no significant differences between the transduction with Ad-IFNB Ad-p14ARF and combination was observed. in this in vitro

MTT assay, all three vectors effectively decrease cellular metabolic activity, regardless of the cell line analysed.

Together the results presented in Figure 8 and 9, points to the existence of a possible advantage of the combined gene transfer of p14ARF and IFNβ over the mono-treatment conditions. What resembles the previous observations of our group regarding the effects of combined gene transfer (152, 171). However, in our current study, between monocistronic treatments it is not clear whether one has a superior effect on OCMCL since the monocistronic vector of major impact varied depending on the molecule or effect evaluated. While the best performing treatment was variable in vitro, the in vivo tests, discussed below, indicate that Ad-IFNβ. Still, despite not able to induce better performance in some of the tests, in vivo tests presented below (Figure 13, 11) demonstrated that, the Ad-INFβ seems to be the more efficient treatment condition.

Evaluation of immunogenic cell death markers

Next, we evaluated the presence of immunogenic cell death markers. Firstly, the secretion of ATP. The results show that the adenoviral vector particles alone are not enough to induce significant cellular stress in both cell lines. In GAB-F6 cells (Figure 12A) transduced by p14ARF encoding vector alone, the ATP detection in culture medium was about 40 times, more than seen in the negative control and GFP conditions. For co-transduced cells, the mean levels of ATP registered were about 80-fold higher. Oddly, BAN-C10 (Figure 12B) did not result in significant alterations between conditions. However, in GAB-F6 a similar pattern, with exception of an elevated level ATP secretion in IFNβ, was observed, although with all conditions below 6-fold the negative control.

In this experiment we observed a marked discrepancy in the release of ATP when comparing the two cell lines. GAB-F6 cells (as depicted in Figure 12A), clearly has the potential to release high amounts of this marker when transduced with either Ad-ARF or Ad-IFNβ alone. Even more expressive is the ATP release induced by the co-transduction these vectors in comparison to the other conditions (Figure 12A). In contrast, no significant different among conditions was observed in BAN-C10 line. Nonetheless, an observable tendency towards higher ATP secretion was identified. However, it remains uncertain whether these inclinations represent real tendency or merely a coincidental occurrence.

Therefore, this assay showed that in at least one cell line, the treatment, especially co-transduction, leads to the liberation of ATP. This may contribute to immune responses since

it is associated with certain types of ICD. The ATP release by dying tumour cells is another key element in the context of immunogenic cell death. This process involves the activation of various signal transduction pathways to facilitate the release of cytoplasmic ATP from the dying cancer cells. Additionally, it functions as a “find me” signal able to attract myeloid cells, particularly dendritic cells, to the tumour (172).

In regards to the release of HMGB1 (Figure 12C), we observed that both cell lines suffered some level of induction of HMGB1 release. In the case of BAN-C10, the release of HMGB1 was detected in cells transduced with IFNβ, p14ARF, and those co-transduced. Conversely, in GAB-F6, the presence of HMGB1 was evident solely in cells transduced with p14ARF and in the co-transduction condition.

In summary, both cell lines released at least one ICD marker upon viral treatment, indicating the possibility of an immune induction after treatment. To further evaluate these findings, we believe it would be interesting to check additional ICD markers such as calreticulin exposure, annexin A1, and tumour cell-derived nucleic described by the Nomenclature Committee on cell death 2018 (158). Note that IFNβ provided by the treatment is also an ICD factor. Current efforts are focussed on the detection of calreticulin exposure on cellular membranes. The qPCR assays, currently in progress, may also shed some light on the pro-immunogenic cellular response to treatment.

In vivo xenograft mouse model of oral canine melanoma cell lines

Afterwards, in an effort to validate the potential of our viruses to impede tumour growth, as already demonstrated in murine tumours through prior research of our group, we initiated a xenograft mouse model involving tumours derived from canine cells. In pursuit of this objective, we introduced 1x10^6 GAB-F6 and BAN-C10 cells s.c. into BALB C nude mice. Once the tumours attained a discernible palpable size, we administered treatments. Four intratumoral inoculations of $1x10^9$ viable viral particles were given to each mouse, each other day. Animals received PBS and a virus carrying eGFP as a control, and treatments condition under analysis (Ad-ARF Ad-IFNβ and co-transduction). Subsequently, we proceeded with the monitoring of the response of the animals to the treatment.

To compare the treatments, analysing a time to outcome (tumour development to $400mm^3$), at the moment when GFP and NC groups reached around $400mm^3$, tumours of the co-transduction group were about 50 mm^3. This shows efficacy of co-transduction to impede tumour development in both cell lines, in comparison to the other conditions. The survival

curve, as illustrated in Figure 13, demonstrates an intriguing pattern. Animals treated with IFN-β and the combination therapy exhibit survival for an additional 20 days after animals subjected to other treatments have already reached a tumour size of 1000mm³, and therefore subjected to euthanasia. This may indicate that in accordance with other models studied in our group, in OCMCL IFN-β and combination also presents better performance in vivo. Nonetheless, the complete efficiency of the combination of genes may not induce better results in this model, what may be related to this model limitation due to the immunodeficiency of the mice.

Regarding the other OCMCL BAN-C10, similar delay on tumour development was observed in the initial days after treatment. In BAN-C10, after the medium tumour size of NC and GFP groups reach the cut-off of about 400 mm^3, the ARF group was around 100 mm^3 and IFNβ and co-transduction groups were 50 and 25 mm^3, respectively. In the Kaplan Meyer analysis, mice maximum lifespan did not present great differences among these conditions and of the controls. Nonetheless, a larger number of animals in the IFN-β and combination groups survived longer.

It is important to remember that the that the model used for this assay involved BALB/c nude mice, which are recognized as immunosuppressed animals. Despite their immunosuppressed status, they still retain a degree of Natural Killer (NK) cell activity (15), but it apparently was not sufficient to completely eliminate the tumour. Yet, these assays have demonstrated that the vectors themselves already have a strong antitumoral effect, likely attributed to the cytotoxicity induced by the transgenes they carry. Histopathology and immunohistochemical detection of critical markers of cellular response to treatment are currently underway.

To sum up, using the novel AdRGD-PG vectors, the combined p14Arf and IFNβ gene therapy approach induces high levels of cell death, release some expected ICD markers and also inhibits tumour progression in a xenograft mouse model. However, the impact of these vectors on an immune competent model could not be tested. As the continuation of this work, our group will soon start initial tests on spontaneous cases of oral melanoma in companion dogs in order to better understand the effects of our vectors on the activation of immune responses.

6. FINAL CONSIDERATIONS AND PERSPECTIVES

Through the findings presented here, we have demonstrated the functional impact of the transduction of p14ARF and IFNβ cDNA using adenoviral vectors in canine cell lines. Moreover, we verified transgenes expression as well as the induction of a significant increase in hypodiploid cells after treatment of GAB-F6 and BAN-C10 canine melanoma cell lines in vitro. With ATP and HMGB1 detection we demonstrated the possibility of these vectors to induce immunogenic cell death. In all condition of therapeutic cDNA transfer, at least one ICD marker was observed. Results that are in accordance with what we expected based on our experience with the previous models studied in our lab.

At the end of the work, dog-derived xenograft tumours in mice illustrated the effects of p14ARF and IFNβ on the delay of the tumour development. IFNβ, together with the combined treatment, being the therapeutic modalities with the greatest capacity to increase survival for animals. Also, due to the limitation of the xenograft mouse model, we believe that the effect of these vectors, especially in co-transduction would be higher if injected in fully immune-competent organisms.

With our findings we believe we achieved the goal of this work in validate the novel developed vectors, containing canine p14ARF and IFNβ cDNA, for future research in dogs. Now our group hopes to start tests in dogs soon and we expect that this new veterinary clinical study may be a proof of concept for future human clinical trials.

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8. ATTACHMENTS

Appendix A – CTNBio approval for the execution of this project

8 ISSN 1677-7042 **Diário Oficial da União** - Seção 1 Nº 198, segunda-feira, 15 de outubro de 2018

Extrato Prévio: 6132/2018, publicado no DOU em 29 de agosto de 2018.

Decisão: DEFERIDO

A CTNBio, após apreciação do pedido de parecer para projeto com organismo geneticamente modificado da classe de risco 1 em instalações com nível de biossegurança NB-1, concluiu pelo deferimento, nos termos deste Parecer Técnico.O Presidente do Comissão Interna de Biossegurança do Instituto de Ciências Biomédicas da USP, Dr. Enrique Mario Boccardo Pirulino, solicita à CTNBio parecer técnico para extensão do Certificado de Qualidade em Biossegurança (CQB) da instituição para inclusão da área do Laboratório de Neurogastroenteologia do Departamento de Anatomia, para execução de atividades de pesquisa em regime de contenção e ensino com organismos geneticamente modificados da classe de risco biológico 1 nas instalações da instituição. As instalações a serem credenciadas são denominadas: Laboratório de Neurociências, situado sala 104A, Edifício Biomédicas III, Av. Prof. Lineu Prestes, 2415. Cidade Universitária, Butantan, São Paulo-SP. CEP 05508-000. A instituição solicita que as áreas sejam credenciadas para o nível de biossegurança 1 junto a CTNBio. Os organismos a serem manuseados pela instituição nestas instalações são linhagens de camundongos (Mus musculus) geneticamente modificados da classe de risco 1. O projeto a ser desenvolvido denomina-se: "Papel do receptor P2X7 nos neurônios entéricos e glias entéricas na colite ulcerativa experimental" sob a responsabilidade da Dra. Patricia Castelucci. A responsável pela unidade operativa declara que as instalações contam com salas e equipamentos úteis em nível de biossegurança adequado às atividades propostas. O processo descreve as condições de biossegurança das áreas a serem cadastradas, as medidas de biossegurança propostas para o laboratório e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização dos projetos propostos. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.098/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 215ª Reunião Ordinária da CTNBio, realizada em 05 de setembro de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº 01250.032749/2018-74

Requerente: Departamento de Radiologia e Oncologia - FMUSP. Centro de Investigação Translacional em Oncologia

CQB: 084/98

Endereço: Instituto do Câncer do Estado de São Paulo/ICESP. Centro de Investigação Translacional em Oncologia/CTO. Av. Dr Arnaldo, 251 - 8ºandar. São Paulo - SP. CEP 01246-000.

Assunto: Solicitação de parecer para execução de projeto de pesquisa com organismo geneticamente modificado da classe de risco 2 .

Extrato Prévio: 6134/18 publicado em 29 de agosto de 2018.

Decisão: DEFERIDO

A CTNBio, após apreciação da Solicitação de parecer para execução de projeto de pesquisa com organismo geneticamente modificado da classe de risco 2, concluiu pelo deferimento, nos termos deste Parecer Técnico. A presidente da Comissão Interna de Biossegurança do Departamento de Radiologia e Oncologia - FMUSP, Dra. Maria Aparecida Nagai, solicita à CTNBio parecer técnico para execução de projeto de pesquisa com organismo geneticamente modificado da classe de risco 2 em instalações com nível de biossegurança NB-2. O projeto de pesquisa a ser executado denomina-se: "Modelo canino de imunoterapia de melanoma utilizando vetores adenovirais portadores dos cDNAs de p14Arf e interferon-beta." e será executado nas instalações do Laboratório de Vetores Virais (LVV) do Centro de Investigação Translacional em Oncologia (CTO). Os organismos a serem manipulados nesse projeto são linhagens de células de mamíferos transformadas com vetores adenovirais. O responsável pelo projeto de pesquisa será o Dr. Bryan Eric Strauss e este declara que o laboratório conta com equipamentos úteis para as atividades experimentais em nível de biossegurança adequado. O processo descreve as condições de biossegurança das áreas a serem utilizadas, as medidas de biossegurança propostas para o projeto e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização do projeto proposto. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende as normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.099/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 215ª Reunião Ordinária da CTNBio, realizada em 05 de setembro de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº 01250.032752/2018-98

Requerente: Departamento de Radiologia e Oncologia - FMUSP. Centro de Investigação Translacional em Oncologia

CQB: 084/98

Endereço: Instituto do Câncer do Estado de São Paulo/ICESP. Centro de Investigação Translacional em Oncologia/CTO. Av. Dr Arnaldo, 251 - 8ºandar. São Paulo - SP. CEP 01246-000.

Assunto: Solicitação de parecer para execução de projeto de pesquisa com organismo geneticamente modificado da classe de risco 2 .

Extrato Prévio: 6135/18 publicado em 29 de agosto de 2018.

Decisão: DEFERIDO

A CTNBio, após apreciação do pedido de parecer para projeto com organismo geneticamente modificado da classe de risco 2 em instalações com nível de biossegurança NB-2, concluiu pelo deferimento, nos termos deste Parecer Técnico. A presidente da Comissão Interna de Biossegurança do Departamento de Radiologia e Oncologia - FMUSP, Dra. Ementa: A presidente da Comissão Interna de Biossegurança do Departamento de Radiologia e Oncologia - FMUSP, Dra. Maria Aparecida Nagai, solicita à CTNBio parecer técnico para execução de projeto de pesquisa com organismo geneticamente modificado da classe de risco 2 em instalações com nível de biossegurança NB-2. O projeto de pesquisa a ser executado denomina-se: "Produção de Receptor Quimérico para Antígenos CD19, CD33 ou CD123 (CAR-T 19, 33 ou 123) em células de leucemia eritrocitária K562 para uso terapêutico em pacientes com Leucemia Mieloide Aguda" e será executado nas instalações do Laboratório de Vetores Virais (LVV) do Centro de Investigação Translacional em Oncologia (CTO). Os organismos a serem manipulados nesse projeto são linhagens de células de mamíferos transformadas com vetores lentivirais. A responsável pelo projeto de pesquisa será a Dra. Patricia Bortman Rozenchan e esta declara que o laboratório conta com equipamentos úteis para as atividades experimentais em nível de biossegurança adequado. O processo descreve as condições de biossegurança das áreas a serem utilizadas, as medidas de biossegurança propostas para o projeto e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização do projeto proposto. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.100/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 215ª Reunião Ordinária da CTNBio, realizada em 05 de setembro de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº 01250.034241/2018-19

Requerente: Hospital de Clínicas de Porto Alegre - HCPA

CQB: 0148/2001

Endereço: Rua Ramiro Barcelos, n. 2350. Porto Alegre-RS. CEP 90035-903.

Assunto: Solicitação de parecer para execução de estudo clínico com organismo geneticamente modificado da classe de risco 1.

Extrato Prévio nº: 6137/18 publicado no DOU de em 29 de agosto de 2018.

Decisão: Deferido

A CTNBio, após apreciação da Solicitação de parecer para execução de estudo clínico com Organismo geneticamente modificado da classe de risco 1, concluiu pelo deferimento, nos termos deste Parecer Técnico. O presidente da Comissão Interna de Biossegurança do Hospital das Clínicas de Porto Alegre - HCPA, Dr. Guilherme Baldo, solicita à CTNBio parecer técnico para execução de estudo clínico com organismo geneticamente modificado da classe de risco 1 em instalações com nível de biossegurança NB-1. O estudo de clínico a ser conduzido denomina-se: "Estudo de Fase I/II, Monocêntrico e Aberto para Avaliar a Segurança, Tolerância, Farmacodinâmica e Eficácia Preliminar da Terapia Genética RGX-111 Intracisternal em Participantes com Mucopolissacaridose Tipo I Grave" sob a responsabilidade do Dr. Roberto Giugliani. O responsável pelo estudo clínico declara que a instituição conta com equipamentos úteis para as atividades experimentais em nível de biossegurança adequado. O processo descreve as condições de biossegurança das áreas a serem utilizadas, as medidas de biossegurança propostas para o projeto e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização do projeto proposto. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido não atende plenamente as normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

SECRETARIA DE RADIODIFUSÃO

DEPARTAMENTO DE RADIODIFUSÃO EDUCATIVA, COMUNITÁRIA E DE FISCALIZAÇÃO

PORTARIAS DE 10 DE OUTUBRO DE 2018

A DIRETORA DO DEPARTAMENTO DE RADIODIFUSÃO EDUCATIVA, COMUNITÁRIA E DE FISCALIZAÇÃO no uso da competência que lhe foi delegada por meio da Portaria MCTIC nº 2881, publicada no D.O.U. de 05 de junho de 2017, e tendo em vista o que consta nos processos abaixo, resolve:

Art. 1º Aplicar às Entidades abaixo relacionadas a penalidade de advertência.

Art. 2º Estas Portarias entram em vigor na data de suas publicações.

Nº do Processo	Entidade	Serviço	Município	UF	Sanção	Enquadramento Legal	Portaria	Embasamento da Portaria de Multa
53542.003214/2015	Rádio Princesa Do Sul Ltda	OM	Goiatuba	GO	Advertência	Art. 5º, parágrafo único, da Portaria MC nº 26/1996	Portaria DECEF nº 4713 de 10/10/2018	Portaria MC nº 112/2013
53542.003213/2015	Rádio Pousada Do Rio Quente Ltda	OM	Caldas Novas	GO	Advertência	Art. 5º, parágrafo único, da Portaria MC nº 26/1996	Portaria DECEF nº 4870 de 10/10/2018	Portaria MC nº 112/2013
53516.001825/2015	Rádio Cultura Palotinense Ltda	OM	Palotina	PR	Advertência	Art. 5º, parágrafo único, da Portaria MC nº 26/1996	Portaria DECEF nº 4998 de 10/10/2018	Portaria MC nº 112/2013

INEZ JOFFILY FRANÇA

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Documento assinado digitalmente conforme MP nº 2.200-2 de 24/08/2001, que institui a Infraestrutura de Chaves Públicas Brasileira - ICP-Brasil.

Appendix B – CEUA approval for the use of animals in this research

Faculdade de Medicina da Universidade de São Paulo
Avenida Dr. Arnaldo, 455
Pacaembu – São Paulo – SP

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que a proposta intitulada "**Modelo canino de imunoterapia de melanoma utilizando vetores adenovirais portadores dos cDNAs de p14Arf e interferon-beta.**", registrada com o **nº 1088/2018**, sob a responsabilidade de **Bryan Eric Strauss** e **Otavio Augusto Rodrigues**, apresentada pelo ICESP - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Faculdade de Medicina da USP em reunião de 27.06.18

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	Início: 25-06-2018 Término: 01-02-2022
Espécie/linhagem/raça	Camundongo Balb/c NUDE
Nº de animais	360
Peso/Idade	6 semanas
Sexo	fêmeas
Origem	Biotério FMUSP

A CEUA FMUSP solicita que ao final da pesquisa seja enviado Relatório com todas as atividades.

CEUA-FMUSP, 27 de Junho de 2018

Dr. Eduardo Pompeu
Coordenador
Comissão de Ética no Uso de Animais

Comissão de Ética no Uso de Animais da FMUSP
e-mail: ceua@fm.usp.br

Appendix C – Curriculum Summary (Súmula curricular)

Name: Otavio AugustoHRodrigues

Education

(2018 - 2023) - Faculdade de Medicina da Universidade de São Paulo. PhD candidate, Oncology;

(2013 - 2017) - Pontifícia Universidade Católica do Paraná Bachelor of Biotechnology, Biotechnology.

Complementary education and courses

(2020) - HMX Pro program (Harvard Medical School online learning platform) – [Immunology - Immuno-oncology].

(2017 – 2017). Stem Cells: From Basic Biology to Regenerative Medicine. Carlos Chagas Institute, ICC, Curitiba, Paraná, Brazil.

(2016 – 2016). Cancer genetics (A Genética do Câncer). Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Paraná, Brazil.

Experience

(2017 - 2023) Instituto do Câncer do Estado de São Paulo (ICESP)

(2016 - 2016) Embrapa Florestas - internship

(2015 - 2016) Instituto Carlos Chagas - Fiocruz/PR (ICC/FIOCRUZ) - internship

Publications

RODRIGUES, O. A.; E. Strauss, Bryan . Terapias Alvo Dirigidas, Gênicas e Oncolíticas. In: Roger Chammas, Maria Aparecida Koike Folgueira, Luisa Lina Villa. (Org.). Oncologia Da molécula à clinica. 1ed.São Paulo: Editora dos Editores, 2022, v. 1, p. 423-446.

L.D. Cerqueira, Otto ; Rolemberg Oliveira Silva, Gissele ; de Luna Vieira, Igor ; Vinícius Gomes Lana, Marlous ; Gimenez, Nadine ; Augusto Rodrigues, Otavio ; Roberto Del Valle, Paulo ; Andrade Mendonça, Samir ; E. Strauss, Bryan . Gene-based Interventions for Cancer Immunotherapy. In Vivo and Ex Vivo Gene Therapy for Inherited and Non-Inherited Disorders. 1ed.London: IntechOpen, 2018, v. 1, p. 1-30.

HEISLER, A.M.J ; WERNER, M. S. ; RODRIGUES, O. A. . Biotecnologia e Bioética. In: Agnor Sganzerla; Marcia Regina Chizini Chemin; Patricia Maria Forte Rauli. (Org.). Bioética nas Profissões: ciências da saúde e áreas afins - Série Bioética. Volume 10. 1ed.Curitiba: CRV, 2019, v. 10, p. 201-216.

ROLEMBERG Oliveira Silva, Gissele; RODRIGUES, O. A.; E. Strauss, Bryan. Establishment of canine melanoma cell lines, molecular characterization, and development of a model of cancer gene therapy. In: Second AACR Conference: Translational Cancer Medicine, 2018, São Paulo. Program and Proceedings. Philadelphia: AACR, 2018. v. 1. p. 28-28.

4.
RODRIGUES, O. A.; HEISLER, A.M.J ; Cunha, T.R. ; SIMÃO, D. P. . Terapia gênica Hereditária: igualdades e desigualdades na sociedade futura.. In: Daiane Priscila Simão-Silva; Leo Pessini. (Org.). Bioética Tecnologia e Genética. 1ed.Curitiba: CRV, 2017, v. 5, p. 33-50.

Links:

Orcid (https://orcid.org/my-orcid?orcid=0000-0003-3938-8076)

Linkedin (https://www.linkedin.com/in/otavio-augusto-rodrigues/)

Appendix D – Article published in the journal doi: 10.3390/cancers13081863.

Review

Nonreplicating Adenoviral Vectors: Improving Tropism and Delivery of Cancer Gene Therapy

Nayara Gusmão Tessarollo, Ana Carolina M. Domingues, Fernanda Antunes, Jean Carlos dos Santos da Luz, Otavio Augusto Rodrigues, Otto Luiz Dutra Cerqueira and Bryan E. Strauss *

Viral Vector Laboratory, Center for Translational Investigation in Oncology, Cancer Institute of São Paulo/LIM24, University of São Paulo School of Medicine, São Paulo 01246-000, Brazil; nayara.tessarollo@hc.fm.usp.br (N.G.T.); ana.domingues@fm.usp.br (A.C.M.D.); fernanda.antunes@hc.fm.usp.br (F.A.); jc.santosluz@usp.br (J.C.d.S.d.L.); otavio.rodrigues@hc.fm.usp.br (O.A.R.); ottoluiz@usp.br (O.L.D.C.)

* Correspondence: bstrauss@usp.br or bryan.strauss@hc.fm.usp.br

Simple Summary: The treatment of cancer has progressed greatly with the advent of immunotherapy and gene therapy, including the use of nonreplicating adenoviral vectors to deliver genes with antitumor activity for cancer gene therapy. Even so, the successful application of these vectors may benefit from modifications in their design, including their molecular structure, so that specificity for the target cell is increased and off-target effects are minimized. With such improvements, we may find new opportunities for systemic administration of adenoviral vectors as well as the delivery of strategic antigen targets of an antitumor immune response. We propose that the improvement of nonreplicating adenoviral vectors will allow them to continue to hold a key position in cancer gene therapy and immunotherapy.

Citation: Tessarollo, N.G.; Domingues, A.C.M.; Antunes, F.; Luz, J.C.d.S.d.; Rodrigues, O.A.; Cerqueira, O.L.D.; Strauss, B.E. Nonreplicating Adenoviral Vectors: Improving Tropism and Delivery of Cancer Gene Therapy. *Cancers* **2021**, 13, 1863. https://doi.org/10.3390/cancers13081863

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Abstract: Recent preclinical and clinical studies have used viral vectors in gene therapy research, especially nonreplicating adenovirus encoding strategic therapeutic genes for cancer treatment. Adenoviruses were the first DNA viruses to go into therapeutic development, mainly due to well-known biological features: stability in vivo, ease of manufacture, and efficient gene delivery to dividing and nondividing cells. However, there are some limitations for gene therapy using adenoviral vectors, such as nonspecific transduction of normal cells and liver sequestration and neutralization by antibodies, especially when administered systemically. On the other hand, adenoviral vectors are amenable to strategies for the modification of their biological structures, including genetic manipulation of viral proteins, pseudotyping, and conjugation with polymers or biological membranes. Such modifications provide greater specificity to the target cell and better safety in systemic administration; thus, a reduction of antiviral host responses would favor the use of adenoviral vectors in cancer immunotherapy. In this review, we describe the structural and molecular features of nonreplicating adenoviral vectors, the current limitations to their use, and strategies to modify adenoviral tropism, highlighting the approaches that may allow for the systemic administration of gene therapy.

Keywords: nonreplicating adenovirus vector; cancer; gene therapy; routes of delivery; virus coated with cancer cell membrane

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1. Overview: Structural and Molecular Features of Nonreplicating Adenoviral Vectors

Adenoviruses (Ads) are one of the most well-studied and widely used viral vectors, representing 17.5% (n = 573) of vectors used in gene therapy clinical trials [1]. The first gene therapy was approved in 2003 by the China Food and Drug Administration. Gendicine is a recombinant nonreplicating adenovirus encoding human p53 and, despite more than 17 years of commercial use, it has only been tested in clinical trials in China for the treatment of hepatocellular, nasopharyngeal, gastric, liver, lung, breast, prostate, ovarian, and head and neck cancer, either alone or in combination with radio- or chemotherapy [2]. This

therapy serves to illustrate the potential for using nonreplicating adenoviral vectors as part of effective cancer treatment.

Recently, nonreplicating adenoviral vectors have gained attention due to their use in the development of vaccines, especially to combat SARS-CoV-2, the novel coronavirus. These vaccines include those based on recombinant adenovirus serotypes, such as human adenovirus vector 5 [3–7], chimpanzee adenovirus vector ChAdOx1 [8,9], and combined human serotypes vectors 5 and 26 [10,11]. The Ad vectors can elicit robust and durable cellular and humoral immune responses [12]. The induction of a balanced innate immune response makes the Ad vectors good candidates for vaccine platforms, and they can also play a role in cancer gene therapy.

Replicating adenoviral vectors are used for the induction of oncolysis (also referred to as oncolytic adenovirus or virotherapy), and these vectors have played a major role in showing the potential of adenoviruses in cancer immunotherapy. The use of nonreplicating adenoviral vectors also deserves particular attention. We argue that nonreplicating vectors perform quite well and may even offer advantages when compared to the use of their replicating counterparts, especially concerning the delivery of proimmune but antiviral transgenes. While we do not discount oncolytic viruses, we do support the continued development of nonreplicating adenoviral vectors for cancer immunotherapy. As shown in Table 1, many clinical trials that are underway involve the use of nonreplicating adenoviral vectors for cancer gene therapy. In this review, we focus on nonreplicating adenoviral vectors, discussing vector biology and current barriers to cancer gene therapy. We also propose some strategies that enhance vector performance, especially in terms of virus delivery and targeting, thus supporting the use of nonreplicating adenoviruses for cancer immunotherapy.

Table 1. Clinical trials using nonreplicating adenoviral vectors for cancer gene therapy.

Vector	Transgene	Cancer	Mechanism	Therapy	Phase	Clinical Trial/ Reference	Status
Ad5-SGE-REIC/Dkk3	REIC/Dkk3	Localized prostate cancer	Cancer cell death induction and anticancer immunity	Neoadjuvant	I/II	NCT01931046 [13] #	Active, not recruiting
Ad5-SGE-REIC/Dkk3 (MTG201)	REIC/Dkk3	Relapsed malignant pleural mesothelioma	Cancer cell death induction and anticancer immunity	Combination with nivolumab	II	NCT04013334 [14] #	Active, recruiting
AdHSV-tk /GCV	HSV-tk Ad-hCMV-Flt3L	High-grade malignant gliomas	TK: direct tumor cell killing Flt3L: immunostimulating effects		I/II	NCT01811992 [15] #	Active, not recruiting
Adv/tk (GMCI)	HSV-tk	Advanced nonmetastatic pancreatic adenocarcinoma	TK: direct tumor cell killing	Neoadjuvant plus chemoradiation	II	NCT02446093	Active, not recruiting
Adv/tk	HSV-tk	Advanced hepatocellular carcinoma	TK: direct tumor cell killing	Liver transplantation	III	NCT03313596 [16] #	Active, recruiting
Adv/tk (GMCI)	Adv-tk	Pediatric brain tumors	Direct tumor cell killing	Combination with radiation therapy	I	NCT00634231 [17] #	Active, not recruiting
Adv/RSV-tk	HSV-tk	Recurrent prostate cancer	Direct tumor cell killing	Combination with brachytherapy	I/II	NCT01913106	Active, recruiting

Table 1. *Cont.*

Vector	Transgene	Cancer	Mechanism	Therapy	Phase	Clinical Trial/ Reference	Status
Adv/HSV-tk	HSV-tk	Metastatic nonsmall cell lung carcinoma and uveal melanoma	Direct tumor cell killing	Combination with stereotactic body radiation therapy or nivolumab	II	NCT02831933	Terminated (Lack of funding)
Ad/PNP + fludarabine	PNP	Head and neck squamous cell carcinoma	PNP protein actives the second component of the therapy fludarabine phosphate		I	NCT01310179 [18]	Completed
rAd-IFN/Syn-3 (Instiladrin)	IFN α-2b	High-grade nonmuscle invasive bladder cancer	Immunoregulatory effects		III	NCT02773849 [19] #	Active, not recruiting
BG00001	IFN-β	Pleural mesothelioma	Immunoregulatory effects		I	NCT00299962 [20]	Completed
Ad-RTS-hIL-12	IL-12	Advanced or metastatic breast cancer	Proinflammatory cytokine, enhances the cytotoxic activity of T-lymphocytes and resting natural killer cells	Combination with VELEDIMEX	Ib/II	NCT02423902	Unknown
Ad-RTS-hIL-12	IL-12	Recurrent or progressive glioblastoma	Proinflammatory cytokine, enhances the cytotoxic activity of T-lymphocytes and resting natural killer cells	Ad-RTS-hIL-12 + Veledimex in combination with Cemiplimab	II	NCT04006119	Active, not recruiting
Ad-RTS-hIL-12	IL-12	Glioblastoma or malignant glioma	Proinflammatory cytokine, enhances the cytotoxic activity of T-lymphocytes and resting natural killer cells	Combination with Veledimex	I	NCT02026271 [21] #	Active, not recruiting
SCH-58500	P53	Primary ovarian, fallopian tube, or peritoneal cancer	Tumor suppressor gene: antitumor effect by blocking cell cycle progression at the G1/S, activating DNA repair pathways		I	NCT00002960 [22]	Completed
Ad-p53	P53	Recurrent or metastatic head and neck squamous cell carcinoma	Tumor suppressor gene: antitumor effect by blocking cell cycle progression at the G1/S, activating DNA repair pathways	Adjuvant in combination with Anti-PD-1 or Anti-PD-L1 therapy	II	NCT03544723	Active, recruiting
ADVEXIN	P53	Squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx	Tumor suppressor gene: antitumor effect by blocking cell cycle progression at the G1/S, activating DNA repair pathways		I/II	NCT00064103 [23]	Completed

partial outcomes.

Human adenoviruses (HAds) are subdivided into seven species (A–G) and >50 serotypes based on serological properties, DNA homology, genome organization, and oncogenicity [24]. More than 100 types of human adenovirus and >200 nonhuman Ad serotypes have been identified to date [25].

Ads carry a linear double-stranded DNA genome (26 to 46 kb in length) and core proteins inside an icosahedral capsid [26]. The Ad DNA genome contains two inverted terminal repeats (100–140 bp) and can be divided into five early genes and five late genes. The capsid facets are formed by structural proteins, mainly composed of hexons, and each vertex contains a penton base that anchors the trimeric protein fiber, divided into the fiber knob and shaft. The viral particles have around one million amino acid residues (weight around 150 MDa) and an average size of 90–100 nm [27]. In the infection process, the knob interacts with cell surface receptors such as the coxsackievirus and adenovirus receptor (CAR), CD46, CD80/86, and desmoglein 2 (DSG2) [28,29]. This interaction leads to viral particle immobilization, which facilitates the interaction between the penton base and integrins [30] and, thus, virion internalization. The DNA and some core proteins are transported through the microtubular complexes to the nuclear pore and are introduced into the cell's nucleus [31]. Inside the nucleus, the viral DNA remains episomal, and the expression of the early genes (E1A, E1B, E2, E3, and E4) suppresses transcription from the host genome, thus favoring adenovirus protein synthesis and replication. Then, the late genes (L1–L5) are expressed, leading to virus encapsulation and viral particle maturation in the nucleus during the completion of the lytic cycle. Nuclear and cytoplasmatic membranes are disrupted, and new virions are released from permissive cells 48–72 h after infection [32,33].

Many characteristics of adenoviruses, such as their safety, broad cell tropism, and ability to stimulate a robust immune response, favor their use as a viral vector platform employed as a gene delivery tool in gene therapy, as an oncolytic cancer treatment, and in the development of vaccines [12,32]. Moreover, the genome of Ads is well characterized, genetically stable, and does not integrate into the host's genome but remains as episomal DNA in the cell nucleus. In addition, adenoviral vectors are modified to control viral replication, have a large cloning capacity (up to 37 kb), can transduce both dividing and quiescent cells, and have high in vivo transduction efficiency [34–36]. Human adenovirus type 5 (HAd5) is the most frequently used adenovirus for the development of gene therapy vectors, which promote the expression of transgenes in the target cells yet have impaired replication and, hence, prevent unwanted virus spread. There are three generations of nonreplicating adenoviral vectors used in gene therapy. In the first generation, E1 and E3 early genes were deleted, rendering vector replication defective but maintaining the ability to transduce host cells without killing them and liberating ~8 kb of space in the genome for the genetic payload (transgene(s) plus regulatory sequences) [37,38]. Since the E1 region is essential for virus replication, E1A proteins induce the transcription of Ad genes, and E1B proteins inhibit cellular apoptosis. Vector production requires that the E1 gene be supplied by transcomplementation, using cell lines (such as HEK293 or PERC.6) that were modified to incorporate the viral E1 region [39,40]. For the second-generation adenoviral vectors, beyond E1/E3 deletions, E2 or E4 regions have also been removed, providing additional space for cargo sequences (~10.5 kb). Third-generation adenoviral vectors were generated after deletion of almost all viral sequences except for the ITRs, the packaging signal, and minimal sequences required for genome replication and encapsulation during vector production [41].

Therefore, nonreplicating adenoviral vectors, different from their replicating counterparts, do not provoke the same cellular responses due to their lack of viral protein expression, absence of viral genome replication, and deficiency in the ability to induce cytopathic effects.

2. Current Applications of Nonreplicating Adenoviral Vectors in Cancer Immunotherapy

There are two main routes to delivering gene therapy vectors: ex vivo and in vivo. In vivo gene transfer raises concerns related to the specificity of vector transduction and transgene transcription in the intended target cells in order to achieve the desired therapeutic outcome, a goal that may be compromised by off-target effects. Ex vivo gene transfer occurs outside the body, where the patient's cells are modified and reinfused. Here, we focus on the in vivo route, particularly the challenges associated with the antiviral immune response.

In general, gene therapy approaches that overcome the immunosuppressive tumor microenvironment (TME) and activate an antitumor immune response are expected to function as cancer immunotherapies. To this end, adenoviral vectors have been modified with a variety of immune-stimulating genes, such as cytokines, costimulatory molecules, tumor-associated antigens, and tumor-suppressor genes [42]. The purpose is not only the direct killing of tumor cells but also the activation of immune cells to attack the tumor. Thus, gene therapy may induce immunogenic cell death and/or the liberation of factors that will then go on to promote the immune response.

The adenoviral vector itself is expected to participate in the activation of an antiviral response that may be both an asset and a complication for gene therapy since attracting the immune response to the tumor site is desirable but the inhibition of viral activity may thwart treatment. After viral entry, pathogen-associated molecular patterns (PAMPs), including viral nucleic acids and viral capsids, are recognized by pattern recognition receptors (PRRs) and activate antiviral immune responses that result in the production of type-I interferons (IFNs), proinflammatory cytokines, and chemokines.

Another important signaling cascade stimulated by the interaction of the virus with CAR and αv integrins is nuclear factor-kB (NF-kB), which mediates the expression of chemokines and interleukin (IL)-1 [43]. Inside the cell, viral DNA is sensed by several cytosolic PRRs such as Toll-like receptor (TLR)-9 [44], DNA-dependent activator of IFN-regulatory factors (DAIs) [45], cytosolic inflammasomes (NALP3) [46], and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors (NLRs)) [47]. As a result, a signaling cascade is initiated, either dependent or independent of myeloid differentiation primary response gene 88 (MyD88), which culminates in the transcription factor (NF-kB, IRF3, IRF7)-mediated expression of IFN-α, IFN-β, and IL-6, among other proinflammatory cytokines and chemokines. In turn, the immunosuppressive TME is modulated to facilitate the recruitment of antigen-presenting cells (APCs) and helper and cytotoxic T-cells. Adenoviral vectors can be especially useful in the treatment of cold tumors [48], which lack immune infiltrate, although the increase in immune cell infiltration may not be enough for the eradication of the tumor [49]. Thus, the approach may be improved if the vector is armed with additional immune-stimulating factors.

Replication-deficient adenoviral vectors have been employed as vaccines and in cancer gene therapy due to strong humoral and T-cell responses to transgenes expressed by the vector [50]. Tatsis et al. (2007) showed that the application of replication-defective adenoviral vectors resulted in sustained levels of $CD8^+$ T-cells specific for the transgene product and persistent levels of transcriptionally active adenoviral vector genomes at the site of inoculation in the liver and lymphatic tissues [51]. In comparisson, replicating adenoviral vectors mimic natural virus infection, resulting in the induction of cytokines and costimulatory molecules that provide a potent adjuvant effect [52]. Both nonreplicating and replicating Ad vectors have been shown to activate effector $CD8^+$ T-cells and central memory T-cells in treated mice. For this, Osada et al. [53] compared Ad5[E1+]CEA, a replicating adenoviral vector carrying the carcinoembryonic antigen (CEA) with two nonreplicating vectors, Ad5[E1−] and Ad5[E1−, E2b−]. When used for the ex vivo transduction of human dendritic cells (DCs), they found that all three vectors yielded similar infectivity and temporal dynamics of transgene expression. In addition, replicating Ad5[E1+]CEA showed toxicity to DCs, eliciting less maturation of DCs and greater clearance by NK cells.

Moreover, Ad5[E1−] and Ad5[E1−, E2b−] were superior to Ad5[E1+] in their capacity to induce and expand antigen-specific T-cell responses. The results suggest that increased replication of an Ad vector may result in diminished efficacy in this scenario, and the deletion of E1, E2, and E3 genes promoted a superior generation of CEA-specific T-cell responses in mice with pre-existing Ad5 immunity.

In the following discussion, we highlight some of the strategies for using nonreplicating adenoviral vectors as cancer immunotherapies in preclinical and clinical assays.

For example, preclinical outcomes in a prostate cancer model have revealed the benefit of immunotherapy based on a heterologous prime-boost, where the virus is injected as a vaccine with concomitant administration of a PD-1-blocking antibody. Similarly, a ChAdOx1–MVA vaccination strategy (a simian adenovirus, ChAdOx1, with the modified vaccinia Ankara virus, MVA) induced $CD8^+$ T-cell responses to the tumor-specific self-antigen of prostate 1 (STEAP1) in murine models. The combination with the anti-PD-1 antibody improved the survival of the animals since tumors were abolished in 80% of the mice [54].

Our laboratory has developed an adenoviral vector, AdRGD-PG, with improved tropism and transgene expression. By including the RGD motif in the fiber knob, transduction no longer relies on CAR but instead uses integrins, which are more widely distributed. The use of a p53-responsive promoter (called PG) to control transgene expression resulted in high-level expression in the presence of wild-type p53 [55,56]. When used to deliver p19Arf (a functional partner of p53) and IFN-β, we observed cooperation between these genes for the induction of cell death in vitro and in vivo using the mouse model of melanoma, B16–F10 [56,57]. Moreover, only combined gene transfers conferred the emission of immunogenic cell death markers ATP, calreticulin, and HMGB1 [56]. The combination of p19Arf and IFN-β proved to be an effective immunotherapy since we confirmed the participation of natural killer (NK) cells and $CD4^+$ and $CD8^+$ T-lymphocytes in immune protection against B16-F10 tumor progression [58]. Other assays showed that the gene transfer of p19Arf and IFN-β using our nonreplicating Ad vector in the LLC1 mouse model of lung carcinoma was able to induce markers of immunogenic cell death. In situ gene therapy with IFN-β, either alone or in combination with p19Arf, could retard tumor progression, but only the combination approach limited the progression of challenge tumors, thus acting as an in situ vaccine [59]. Thus, the p19Arf + IFN-β gene transfer approach induces oncolysis and immune activation even in the absence of viral replication, functioning as a cancer vaccine and immunotherapy, at least in mice [60,61].

We have taken great care to use different models to demonstrate the functionality of our approach since it involves IFN-β, which is known to have species-specific activities [62]. To examine our approach in human melanoma cell lines, we used the AdRGD-PG backbone to construct vectors encoding the human cDNAs p14ARF and hIFN-β and showed immunogenic cell death characterized by the emission of critical markers in vitro as well as successful ex vivo priming of human T-cells [63].

Most of the clinical trials using nonreplicating Ads are in Phase I/II (Table 1). Kumon et al. [13] have demonstrated in preclinical and clinical data the benefit of in situ Ad-REIC (adenoviral vector carrying the human *REIC/Dkk-3* gene) treatment. In preclinical data, they showed that Ad-REIC induces selective toxicity in response to endoplasmic reticulum stress and IL-7 overproduction by infected normal cells, including cells of the TME. These cells can activate innate immunity, especially NK cells, as well as cytotoxic T-lymphocytes (CTLs). In addition, DCs induced by secreted REIC proteins can present cancer antigens from apoptotic cancer cells and induce tumor-associated antigen-specific $CD8^+$ CTLs. In clinical settings, preliminary outcomes have shown cytopathic effects and tumor-infiltrating lymphocytes in patients with high-risk localized prostate cancer, undergoing radical prostatectomy, who received two ultrasound-guided intratumoral injections at 2-week intervals, followed by surgery six weeks after the second injection.

Another interesting approach is gene-mediated cytotoxic immunotherapy (GMCI), which uses an adenoviral vector expressing the herpes simplex virus (HSV) thymidine ki-

nase (TK) gene (ADV/HSV-TK), followed by an antiherpetic prodrug. The HSV-TK protein has two principal functions: (1) nucleotide analog products of prodrug phosphorylation lead to the death of dividing cancer cells, and (2) TK is a superantigen that stimulates a potent immune reaction [64]. GMCI activates the stimulator of interferon genes (STING) pathway, enhancing the production of proinflammatory cytokines such as IFNs and promoting T-cell activation. The first study using ADV/HSV-TK plus ganciclovir for the treatment of human prostate cancer was conducted by Herman et al. [65]. The patients received a single injection of the vector (10^8 to 10^{11} vector particles) into the prostate gland in the region with the greatest concentration of tumor cells. Not only did the regimen prove safe, with minimal toxicity, but three patients that received 10^9–10^{11} viral particles had a decrease of more than 50% in serum prostate-specific antigen (PSA) levels for periods ranging from 45 to 330 days. The safety and efficacy of GMCI to convert the TME from cold to hot have been noted for studies in different tumor types, including glioma [66], retinoblastoma [67], and mesothelioma [68].

A promising Phase III clinical trial is being conducted on patients with BCG refractory nonmuscle invasive bladder cancer (NMIBC). This disease is an early form of bladder cancer, and the recommended treatment for these patients is the use of intravesical Bacillus Calmette-Guérin (BCG). However, data has shown that around 30% to 50% of cases will recur. The outcomes of BCG-unresponsive patients are poor, and total cystectomy (complete removal of the bladder) is the standard of care for patients who are operative candidates [69].

Alternatively, patients with bladder cancer are treated with nadofaragene firadenovec (rAd–IFN-α2b/Syn3), a replication-deficient recombinant Ad carrying the interferon-α gene, which can have both TRAIL- and non-TRAIL-mediated cytotoxic effects. The patients receive the treatment directly into the bladder using a catheter every three months, and there is elevated interferon production and, consequently, increased exposure to urothelium-enhanced cytotoxic activity. Among 157 patients with carcinoma in situ, 53% of patients achieved a complete response in as early as three months, and about 24% of patients remained free of high-grade recurrence at one year. The outcomes are encouraging and currently awaiting Food and Drug Administration (FDA) approval [70].

As mentioned before, replication-defective Ads are also used as cancer vaccine strategies. GVAX, a GM-CSF gene-modified tumor vaccine, was developed by transducing autologous tumor cells with E1/E3-deleted Ad vectors encoding GM-CSF in autologous tumor cells extracted from each patient. In a phase I/II trial, 33 patients with NSCLCs that were refractory to standard treatment received the GVAX vaccine consisting of $5–100 \times 10^6$ irradiated tumor cells per dose, every 2 weeks. This strategy was shown to be safe, and three patients had radiologically complete responses that lasted for more than six months [71].

Nonreplicating Ads have also been exploited as delivery vehicles in dendritic cells (DCs). Briefly, viral particles processed via proteasome result in the presentation of self and foreign antigens by MHCI and MHCII molecules to both $CD8^+$ and $CD4^+$ T-cells, inducing protective humoral and cellular immunity [72]. A phase I/II clinical trial tested the immune response against a vaccine consisting of autologous DCs obtained from patients, transduced ex vivo with Ads encoding the full-length melanoma antigen MART-1/Melan-A. This study pointed to an increase in $CD8^+$ and $CD4^+$ T-cells in 6/11 and 2/4 metastatic melanoma patients, respectively [73].

Another phase I clinical trial performed on patients with advanced NSCLC showed the induction of systemic tumor antigen-specific immune responses with enhanced $CD8^+$ T-cell infiltration of tumors in 7/13 of patients. The treatment consisted of two intradermal injections of autologous DCs, transduced ex vivo with an Ad vector expressing the CCL21 gene [74]. Similar outcomes have been observed in small cell lung cancer, where 41.8% of patients presented specific anti-p53 immune responses when treated with a vaccine consisting of DCs transduced with an Ad encoding p53 [75]. These positive outcomes are not limited to solid tumors. In a phase II study, acute myeloid leukemia (AML) patients with early molecular relapse received a modified DC vaccine. The DCs were modified

with two tumor-associated antigens (TAAs), survivin and MUC1, plus secretory bacterial flagellin for DC maturation and RNA interference to suppress SOCS1. The complete remission rate was 83% among all relapsed AML patients [76].

TAAs are usually expressed in normal tissues at low levels but overexpressed in tumor cells. Many TAAs have been identified as targets for tumor-reactive T-cells and can be isolated from tumor-infiltrating lymphocytes (TILs) [77]. In contrast, tumor-specific antigens (TSAs) are only encoded in cancer cells as a consequence of somatic mutations that alter the amino acid sequence, resulting in foreign proteins that can be presented to the immune system. Therefore, the neoantigens are less susceptible to the mechanisms of immunological tolerance, comprising an interesting target for vaccination [78]. Thus, gene-based vaccination using Ad vectors as a delivery agent is emerging as one of the most promising approaches for loading antigens (TAA or TSA) onto DCs. Important advantages of this modified DC approach include persistent expression of the antigen that results in activation of $CD4^+$ and $CD8^+$ T-cells and the induction of antibody responses and the natural adjuvant stimulating effect that Ads mediate, which contributes to DC maturation [79].

As mentioned above, ex vivo modification of DCs followed by the reintroduction of these cells in the patient is a standard strategy for these vaccines. Even so, in situ targeting of DCs has been explored using either human or murine cells, though it can often be limited by the patient's pre-existing immunity against the adenovirus [80].

3. Challenges of Using Adenoviral Vectors

Even though the use of nonreplicating adenoviral vectors has shown great promise for cancer immunotherapy, several aspects of the virus and its delivery present barriers to its effectiveness. Ideally, the vector should transfer the gene to the intended cell type without causing undue antiviral host responses. In the following discussion, we present the molecular basis for Ad tropism, the anti-Ad immune responses, and the issues surrounding the systemic administration of Ad vectors. With a thorough understanding of these mechanisms, we can then explore solutions for the challenges that they pose.

3.1. Tissue Tropism

As mentioned previously, most human adenovirus serotypes use CAR as their primary receptor, which is expressed on several cell types, including hepatocytes, myocardiocytes, myoblasts, and epithelial and endothelial cells [51] (Table 2). Additionally, some Ads can bind to CD46, a complement regulatory protein that is present on most nucleated human cells, including hematopoietic stem cells and dendritic cells, as well as the costimulatory molecules CD80 and CD86, present on antigen-presenting cells [81–83]. Different primary receptors such as integrin $\alpha v\beta 5$, heparin sulfate proteoglycans, sialic acid, and DSG2 and GD1a glycans have also been reported to support adenovirus internalization [81,84,85], with different primary receptors influencing the route of intracellular viral traffic [86].

Table 2. Classification and tropism of human adenoviruses.

Classification and Tropism of Human Adenoviruses			
Subgroup	**Serotypes**	**Identified Receptors**	**Tropism**
A	12, 18, 31, 61	CAR	Enteric, respiratory
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55, 66,68, 76–79	CD46, DSG2, CD80, CD86	Renal, ocular, respiratory
C	1, 2, 5, 6, 57, 89	CAR, VCAM-1, HSPG, MHC1-a2, SR	Ocular, lymphoid, respiratory, hepatic
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54, 56, 58–60, 62–65, 67, 69–75, 80–88, 90–103	SA, CD46, CAR, GD1a	Ocular, enteric
E	4	CAR	Ocular, respiratory
F	40, 41	CAR	Enteric
G	52	CAR, AS	Enteric

CAR: coxsackie adenovirus receptor. DSG2: desmoglein-2. GD1a: GD1a ganglioside. HSPG, heparin sulfate proteoglycans. MHC1-a2: major histocompatibility complex-a2. SA: sialic acid. SR: scavenger receptor. VCAM-1, vascular cell adhesion molecule-1. Adapted from [85,87].

Engineered HAd can transduce target cells and are internalized in a similar way to wild-type adenovirus infection. The internalization can be augmented by interactions between an arginine-glycine-aspartate (RGD) motif found in the penton base and integrins. Upon attaching to CAR, the fiber knob disassociates from the capsid, and the exposed penton base interacts with a secondary receptor, usually membrane integrins $\alpha v\beta 3$ or $\alpha v\beta 5$, responsible for virus internalization [86,88], followed by virion endocytosis via integrin-mediated signaling [86,89].

Different subgroups of adenovirus can use different types of integrins as receptors, reinforcing their characteristic cell tropism [89,90]. The virus enters the cell by a clathrin-coated vesicle and is transported in endosomes, where capsid disassembly occurs due to endosome acidity. The virion escapes the endosome and traffics to the nucleus by microtubular complexes, where replication occurs [31,85]. After 2 h, about 40% of the internalized wild-type virions arrive in the nucleus, ready to be transcribed due to their double-stranded genome. Additionally, 48–72 h after infection, nuclear and cytoplasmatic membranes are disrupted, and around 10,000 new virions are released [32].

Different serotypes may favor particular receptors; for example, HAd5 from subgroup C has been shown to utilize CAR for facilitating entry into cells [91], while HAd11 and HAd35 from subgroup B utilize CD46 as their primary receptor [83]. As mentioned, the adenoviral vectors may be chosen due to their inherent tissue-specificity and compatibility with the intended route of administration. For example, when the virus is injected into the brain, tropism for specific cell populations depends on the interaction with CAR, and lower transduction is observed with vectors that bind neither to CAR nor integrins [92]. Even so, HAd5 has been shown to enter cells by CAR-independent mechanisms, including via a hexon–lactoferrin bridge [93,94]

The native tropism of Ads for CAR on the cell surface and the interaction of viral vectors with nontarget tissues can result in toxicity and poor therapeutic efficacy. Thus, viral proteins can be genetically tailored to expand or restrict viral replication, and vector replication machinery can even be modified to augment or restrict viral replication in target cells [49]. Beyond that, nonhuman adenoviruses, such as canine (CAd2), bovine (BAd3), chimpanzee (ChAd1-7, ChAd68), and ovine (OAd7), can also be used to overcome the pre-existing immunity in human patients [95].

3.2. *Pre-Existing Immunity in the Host*

Due to the growing application of adenoviral vectors in gene therapy and vaccines, studies of seroprevalence in global populations are important. However, these studies may be limited by the lack of data from South America, Australasia, and most African countries [96]. Moreover, predominant HAdV types can change over time within a region [97],

and transmission of new strains across continents appears to be frequent. A recent study conducted by Mennechet et al. [96] showed that HAdV-D26 seroprevalence appears to be relatively high in Africa and Asia and low in North America and Europe, while HAdV-B35 seroprevalence is low worldwide. HAdV-C5 is the most common serotype that infects humans, particularly in developing countries [98,99], and it is one the most commonly used adenoviral vectors; thus, the limitations on the applicability of the HadV-C5 vector, due to pre-existing immunity, have led to the construction of novel vectors derived from rare Ad serotypes [100].

HAd serotypes are often associated with specific diseases. For instance, serotypes 2–5, 7, and 21 commonly infect the respiratory tract of individuals [101–104], while serotypes 8, 19, and 37 are responsible for keratoconjunctivitis outbreaks [105–107]. Pharyngoconjunctivitis is often associated with serotypes 3, 4, and 7 [108,109] and acute gastroenteritis with serotypes 40 and 41 [110,111]. Likewise, neurological disorders and obesity seem to have some correlation with adenoviral infections [112–114]. Due to this frequent occurrence of these infections worldwide, humans have extensive preexisting immunity to adenoviruses [115–119].

HAd capsid proteins are very immunogenic, especially the hexon protein [120]. The host's adaptive immunity arm detects the hypervariable regions (HVRs) in the hexon protein and releases serotype-specific neutralizing antibodies (NAs) that appear to block a postentry step [121,122]. Thus, at second contact with the same adenovirus serotype, the host NAs may rapidly neutralize it. Interestingly, at the same time, coagulation factor X (FX) in the blood binds to the hexon protein and activates complements (C4 and C4BP in the classical and alternative complement pathways) against the adenoviruses. In a fair number of tested individuals, it also protected the virions from neutralization by serum components [123]. The HAd5 vector interacts with FX, which, in turn, binds cell surface heparan sulfate proteoglycans on hepatocytes; thus, FX is essential for intravenously injected Ad5 vectors to transduce the liver [124].

Even with FX binding the hexon proteins, anti-knob fiber and anti-penton base antibodies can also prevent the adenovirus from transducing cells [125]. Nevertheless, the HAd species C knob and penton base proteins have also been shown to induce serotype-specific NAs [126].

Beyond antibodies, strong and sustained $CD8^+$ T-cell responses follow adenoviral infections [127]. Up to one-third of circulating T-cells against HAd have been reported to be $CD4^+$ T-cells specific for a hexon epitope conserved between HAd serotypes. Hence, the host's preexisting $CD4^+$ T-lymphocytes might promptly respond to various subsequent adenovirus serotypes in either blood or gut [128].

3.3. *Different Administration Routes and Their Particularities*

Although the administration of lower doses of Ads is well-tolerated, higher doses are known to overstimulate innate and adaptive immune responses, which might result in acute toxicity. For example, with HAd5 at the concentration 1×10^{11} PFU/kg, 70% of hepatocytes and 15% of Kupffer cells expressed transgene three days later [129]. Systemic delivery, which theoretically could solve the issue of reaching metastatic foci, is confounded by sequestration of the virus by the liver and the subsequent antiviral immune response as well as possible liver damage [130]. For example, at doses up to 4×10^{12} vp/kg of HAd5, approximately 98% of the injected virus was found in the liver 30 min after injection [131]. Thus, systemic delivery of adenoviral vectors is associated with dose-dependent toxicity and a high risk of hepatotoxicity. Several studies have shown that the delivery of adenoviral vectors to immunocompetent mice by different routes, such as intravenous [132], intraperitoneal and intratracheal [133] or via direct injection into the pancreas, resulted in the production of neutralizing antibodies, decreasing the effectiveness of a second administration [134].

The relationship between the route of administration and viral load on $CD8^+$ T-cell populations has already been studied. Holst et al. [135] administered adenoviral vectors

encoding β-galactosidase by intravenous or subcutaneous routes and then examined transgene-specific $CD8^+$ T-cells. Independently of the route of administration, doses above 10^9 particles were disseminated systemically. In moderate doses, both routes induced a transient peak of IFN-γ produced by $CD8^+$ T-cells 2 to 3 weeks postinfection. However, with intravenous administration, these cells were only detected in the liver. Additionally, after 2 to 4 months, the systemic immunization created dysfunctional transgene-specific $CD8^+$ T-cells impaired in both cytokine production and in vivo effector functions as well as the accumulation of specific $CD8^+$ T-cells in the spleen. Thus, the most important influence of adenovirus administration on $CD8^+$ T cell response is the route of injection and not the total antigen load [135].

In another study, the intralymphnodal administration of a nonreplicating recombinant adenoviral vector encoding the LacZ reporter gene in canine lymphosarcoma was found to be safe, with no relevant adverse effects. This finding presents the potential for its administration to lymph node metastases in both animal and human models [136]. The examples above demonstrate that efficient gene delivery using adenoviral vectors can be performed without hepatic injury or systemic immunogenicity if off-target effects are avoided. To this end, several strategies have been developed to minimize interactions of the adenoviral vector with the liver and to protect the virus from neutralizing antibodies. Some of these approaches, such as the engineering of adenovirus capsid, hexon, or fiber proteins, use of nonhuman serotypes, and nanoformulation-coated adenoviral vectors will be discussed in more detail below.

4. Strategies to Modify Adenovirus Tropism

Although Ads infect many different types of cells, low (or no) expression of CAR, especially in tumor cells, confounds the attachment step and represents one of the hurdles to gene therapy using adenoviral vectors. Several strategies have been employed to overcome this barrier and redirect the Ads to the intended recipient and, consequently, decrease off-target effects (Figure 1).

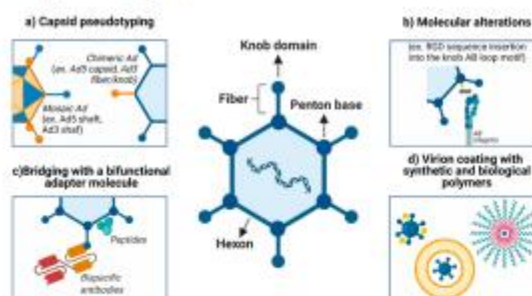

Figure 1. Improvements in vector delivery to and targeting of cancer cells. (**a**) Several approaches can be used to modify viral attachment and entry, such as inhibiting the binding to natural receptors (detargeting) and creating tropism for neoplasms and their metastatic foci (retargeting). (**b**) An alternative to CAR-mediated viral attachment is modifying the fiber (for example, incorporation of the RGD sequence into the knob AB-loop motif). (**c**) Ad structure permits retargeting through the incorporation of synthetic molecules and antibody fragments within the virus capsid. (**d**) Both biological (e.g., cell membrane, liposome) and chemical (e.g., gold, silver, PEG) approaches may be used to coat the virus and improved delivery, especially for the systemic route. PEG: polyethylene glycol. Created with BioRender.com.

4.1. Modifications in Viral Entry: Attachment Receptors and Virus Internalization

To explore the targeting of adenovirus particles to tumor cells, initial events related to infection/transduction must be modulated: (i) viral attachment and (ii) viral entry. This strategy can target tissues by inhibiting binding to natural receptors (detargeting) in normal liver cells, for example, and, simultaneously, creating tropism for neoplasms and their metastatic foci (retargeting) [137]. Another strategy is pseudotyping, the creation of variants by recombining their capsid proteins. Here, we detail works that have used a variety of strategies to modify tropism.

The tropism of HAd5 is predominantly mediated by the interaction of fiber/knob with CAR. As an alternative to CAR-mediated viral attachment, one of the classical fiber modifications is the incorporation of the RGD sequence into the knob AB-loop motif, which greatly expands the spectrum of cell types that may be transduced [138]. Even so, the genetic incorporation of an RGD-4C peptide into the HI loop or the C-terminal end of the HAd5 fiber knob modifies the Ad knob domain without ablating native CAR-binding [139]. Another possibility is the insertion of positively charged polylysine motifs [140]. This modification permits the virus to target the tumor cell's heparan sulfate proteoglycans, common constituents of the cell surface, and the extracellular matrix, overexpressed in several different cancer types, including cervical cancer [141].

On the other hand, directing transduction and expression of the transgenes to occur only in tumor cells (but not in normal cells) should minimize the adverse effects of the therapy. Wickham and coworkers [142] successfully used bispecific antibodies to promote the targeting of an adenoviral vector to endothelial and smooth muscle cells. However, the attempt to noncovalently associate antibodies or molecules with the surface of the viral particle may be hampered by the instability of this binding, especially if used in vivo. For this reason, the adenovirus fiber gene sequence can be edited and, thus, the peptide ligands can be incorporated directly into the protein sequence [137,143–145].

Taking cues from the phage display technique, adenovirus libraries can be generated with random peptide combinations and screened for their ability to transduce a particular cell type, thus refining specificity to tumor populations in a strict manner [146–148]. Joung et al. [148] devised a technique for producing adenovirus with modified fibers that involved cotransfecting a packaging cell with a plasmid encoding a genetically fiber-less adenovirus with a plasmid containing the open reading frames (ORFs) of the fiber of interest. Moreover, Yoshida et al. [144] developed a Cre-lox-mediated recombination system using a plasmid library encoding modified fiber and the adenoviral genome. Using this approach, these authors inserted unique peptides, each with seven random amino acids, into the AB-loop of the fiber, and, after screening, they were successful in targeting these viral vectors to glioma cells [143,149]. Although the idea seems highly promising, there are technical complications that hinder this approach. The compaction and self-assembly of the protein monomers to form the adenovirus particle is a very delicate process. The insertion of random peptides can compromise the final structure of the viral particle as well as virus production [150].

Even though two receptors are required for the adenovirus particle to penetrate the target cells, each interaction is a distinct step. While attachment receptors apparently only recognize the target cell, the HAd5 penton base–αv integrin interaction activates signaling pathways such as p38MAPK [151,152] and Rho GTPases [153], which then trigger changes in the cell cytoskeleton for endocytosis mediated by clathrin [153,154]. Interestingly, mutation of the penton base RGD sequence slows but does not impair virus internalization and infection, nor does it prevent liver tropism [86].

4.2. Pseudotyping the Capsid Using Components from Different Adenoviruses

Chimeric adenoviruses are usually based on HAd5 with the fiber or its knob domain replaced by that of another serotype [155]. This creates perspectives for the recombination of these subtypes and, thus, the modulation of targeting: a concept known as pseudotyping. However, the resulting range of tropisms will be restricted to the respective serotypes used in the construction and may not necessarily contemplate the range of existing receptors

found in neoplasms. Table 3 summarizes important findings in studies that have employed adenovirus pseudotyping strategies.

Table 3. Different studies using adenovirus pseudotyping strategies.

Attachment Receptor	Tropism	Modification	Serotype Origin/ Subgroup	Results	Reference
CD46	Glioma	Fiber replacement	Ad35, Ad16, Ad50	Increased transduction of patient-derived cells	[156]
Adenovirus serotype 3 receptor	Ovarian cancer cells	Fiber knob replacement	Ad3 (modified)/B1	Enhanced gene transfer to various cancer cell lines and primary tumor tissues	[157]
Adenovirus serotype 3 receptor	Lung cancer (NSCLC primary tissue)	Fiber knob replacement	Ad3 (modified)/B1	Improved killing of NSCLC cells	[158]
Sialic acid, phage display for kidney	Renal cancer and detargeting the liver	Fiber knob replacement	Ad5 (modified)/19p (fiber)	Reduced liver tropism and improved gene transfer to renal cancer	[159]
Unidentified cellular receptor	Cancer cell lines of pancreatic, breast, lung, esophageal, and ovarian	Fiber knob replacement	Ad5 (modified)/D49	Efficiently transduced	[160]
CD46	Primary human cell cultures	Fiber replacement	Ad5PTD/F35	Increased transduction capacity of T-cells, monocytes, macrophages, dendritic cells, pancreatic islets, mesenchymal stem cells, and tumor-initiating cells	[161]

PTD: Tat-PTD hexon modification.

4.3. *Encapsulation of Adenovirus Using Synthetic Polymers*

Shielding the virus with nanoparticles allows the Ad to escape immune recognition and avoid the undesirable accumulation of the vector in the liver upon systemic delivery. Furthermore, this approach can enhance the specific targeting of tumor cells. Several studies have been conducted to evaluate the efficacy of encapsulation of negatively charged Ads with cationic liposomes or particles that aim to prevent virus clearance from circulation [162].

Some Ad features, such as regular geometries, well-characterized surface properties, and nanoscale dimensions, make it a biocompatible scaffold for a wide variety of inorganic and biological structures. The Ad capsid has free lysines, the majority of them located on hexon, penton, and fiber proteins, which can be covalently linked to other molecules such as polymers, sugars, biotin, and fluorophores [163]. Polymers offer a wide range of conjugation and encapsulation that make them a safe option for immunotherapy. The main biopolymers studied are polyethylene glycol (PEG) and hydroxylpropyl methacrylamide (pHPMA), the latter being covalently bound to capsid proteins; thus, it can efficiently transduce solid tumors after intravenous injection into mice [164]. The first study using this polymer demonstrated passive tumor targeting of polymer-coated adenoviruses administered by intravenous injection; the authors observed that the coated virus accumulated inside solid subcutaneous AB22 mesothelioma tumors 40 times more than the unmodified virus [164].

As mentioned, Ad structure permits retargeting through the incorporation of synthetic molecules and antibody fragments within the virus capsid. For instance, PEG is an uncharged, hydrophilic, nonimmunogenic, synthetic linear polymer (CH_2CH_2O repetitions) [165] that is frequently utilized in the biopharmaceutical industry and can be useful to protect therapeutic molecules from proteolysis as well as humoral and cellular immune responses [166]. According to Fisher et al. [164], one advantage of vector PEGylation is the retention of viability after storage at various temperatures compared to conventional Ads. Covalent attachment of PEG to the adenovirus capsid may be achieved by using PEG activation mechanisms. PEG presents hydroxyl groups (OH) that make PEG-protein bonds impossible; thus, it is necessary to use chemical activation before pro-

tein attachment. In the specific case of adenoviruses, activation can be achieved through the use of tresyl-monomethoxypolyethylene glycol (TMPEG), succinimidyl succinate-monomethoxypolyethylene glycol (SSPEG), or cyanuric chloride-monomethoxypolyethylene glycol (CCPEG), which react preferentially with lysine residues in the capsid, thus supporting the formation of covalent bonds with PEG [167].

In addition to the PEGylation of the virus particle, ligands can also bind to the opposite extremity of PEG, thus providing a specific ligand to retarget the virus to the corresponding cellular receptor.

Such approaches also aid the vector in reaching distant tumor sites, as found by Eto et al. [167]. They used a cationic liposome that was composed of (1, 2-dioleoyloxypropyl)-N, N, N-trimethy-lammonium chloride:cholesterol to encapsulate the Ad vectors carrying the antiangiogenic gene (pigment epithelium-derived factor (PEDF)). The results showed that systemic administration of Ad-PEDF/liposome was well tolerated and caused the suppression of tumor growth. The coated Ad-PEDF increased apoptosis compared to uncoated Ad in the B16-F10 melanoma cell line and inhibited murine pulmonary metastasis in vivo. Moreover, Ad-luciferase encapsulated with liposome exhibited decreased liver tropism and increased transduction in the lung. Additionally, the anti-Ad IgG level after administration of the Ad-PEDF/liposome was significantly lower compared to Ad-PEDF alone. Eto et al. [167] showed that positively charged 14-nm gold nanoparticles increased the efficiency of Ad infection in mesenchymal stem cells, usually refractory to Ad transduction, mainly because CAR expression is absent or downregulated. The strategies described here support future exploration of additional formulations for liposome-encapsulated adenoviruses and their ability to target cancer cells.

4.4. Cancer Cell Membrane-Coated Adenoviral Vectors

Nanoparticle-based delivery systems have been extensively explored for improving cancer treatment. Cell membranes, which can be obtained from a variety of source cells, including leukocytes, platelets, red blood cells, and cancer cells, are being employed to encapsulate particles such as liposomes, polymers, silica, and Ad vectors in order to improve tumor-targeted drug delivery in addition to prolonged circulation time, reduced interaction with macrophages, and decreased nanoparticle uptake in the liver [168]. The membrane-based functions of cancer-related cells include extravasation, chemotaxis, and cancer cell adhesion [169]. As a source of cell membranes, cancer cells offer certain advantages. They can be obtained from cell lines or patient samples and possess a wide range of membrane surface proteins, such as MHC, TAAs, and neoantigens, that can program the immune system to attack local and distant tumor sites [170], as represented in Figure 2.

Tumors frequently develop a variety of mechanisms to subvert immune attack, resulting in an immune-suppressive TME. Although tumor cells can stimulate a variety of cell types, including fibroblasts, immune-inflammatory cells, and endothelial cells, through the production and secretion of stimulatory growth factors and cytokines [171], the TME can be modulated by the tumor cells themselves and tumor-infiltrating leukocytes (including regulatory T-cells (Tregs)), myeloid-derived suppressor cells (MDSCs), and alternatively activated (type 2) macrophages (M2), cytokines (IL-10, TGF-β), expression of inhibitory receptors (such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-ligand 1 (PD-L1)) or impediment of T-cell function, resulting in the reduced effectiveness of immunotherapy [172]. Although many TAAs have been identified, their immunogenicity is generally insufficient to elicit potent antitumor responses. Typically, when the tumor reaches the malignant stage, the most immunogenic tumor-specific antigens have been eliminated via negative selection. Frequently, the nanoparticles are associated with adjuvants, secretory cytokines, antibodies, and/or viral vectors to improve the immune response [173].

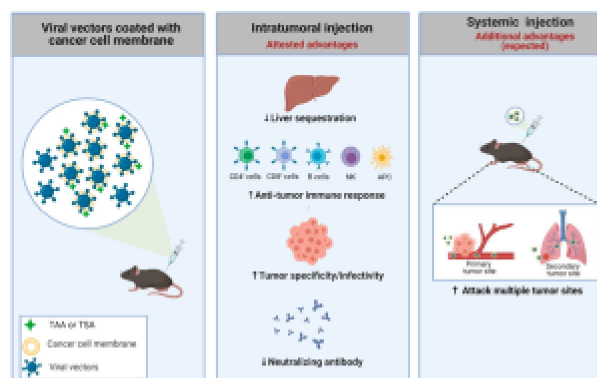

Figure 2. Strategy used to improve the specificity of adenoviral vectors. An adenovirus coated with a cancer cell membrane has some advantages, such as the presence of TSA and TAA, which aids the anti-tumor immune response. Additionally, the membrane can be engineered to present specific molecules/receptors, improving the power of interaction with the tumor. Moreover, the viral coating can offer several benefits, including suppression of liver toxicity, increase of specific infectivity to cancer cells, preferential antitumor (not antiviral) immune response, and escape from pre-existing neutralizing antibodies in both routes of delivery (intratumoral and systemic). Systemic administration using virus coated with membranes could offer a highly desirable outcome: targeting metastatic foci. TAA: tumor-associated antigens; TSA: tumor-specific antigens. Created with BioRender.com.

Coating polymeric nanoparticles with cancer cell membranes can be used for different types of cancer therapy. For anticancer drug delivery, Zhuang et al. [173] showed that polymeric nanoparticle cores made of poly(lactic-co-glycolic acid) (PLGA), a polymer coated in an MDA-MB-435 membrane, significantly increased cellular adhesion to the source cells compared to naked nanoparticles due to a homotypic binding mechanism. For cancer immunotherapy, the authors demonstrated that a polymer coated with a B16−F10 membrane, which creates a stabilized particle, facilitated the uptake of membrane-bound tumor antigens and, consequently, the presentation and maturation of DCs. Another approach using a biohybrid (tumor-membrane-coated) nanoparticle was also able to elicit an antitumor immune response in melanoma models, changing the microenvironment profile. The administration of the vaccine enhanced the activation of APCs and increased the priming of $CD8^+$ T-cells. When combining the nanovaccine with a checkpoint inhibitor, 87.5% of the animals responded, including two complete remissions, when compared to the immune checkpoint inhibitor alone. These results point to opportunities for the association of nanoparticles and immunomodulators to enhance T-cell responses [174].

The effects from the association of polymers, cancer cell membranes, and adjuvants were also observed by Fontana et al. [175]. PLGA nanoparticles were loaded with the TLR7 agonist and then coated with membranes from B16-OVA cancer cells since the presentation of foreign peptide OVA permits the tracking of responses. The nanovaccine was able to enhance uptake by antigen-presenting cells and showed efficacy in delaying tumor growth as a preventative vaccine besides displaying activity against established tumors when coadministered with the anti-programmed death 1 (PD-1) monoclonal antibody.

In another study, CpG oligodeoxynucleotide (CpG) was used as an immunological adjuvant and encapsulated into PLGA nanoparticle cores coated with membranes derived from B16–F10 mouse melanoma cells. The effect of nanoformulation on DC maturation was observed by the upregulated expression of costimulatory markers CD40, CD80, CD86,

and MHC-II. Both prophylactic and therapeutic vaccines presented positive results. In the prophylactic study using the poorly immunogenic wild-type B16–F10 model, tumor occurrence was prevented in 86% of mice 150 days after challenge with tumor cells. Interestingly, mice vaccinated with the CpG-nanoformulation alone had tumor growth comparable to the control group and a median survival of 22 days. This reinforces the role of the cancer cell membrane in targeting the elimination of malignant cells by the immune system. In the therapeutic model, mice challenged with B16–F10 cells and subsequently treated with the nanoformulation presented a modest ability to control tumor growth. However, the combination of nanoformulation and a checkpoint blockade cocktail (anti-CTLA4 and anti-PD1) significantly enhanced tumor growth control. As such, the results encourage further research into nanoparticle vaccine formulation and possible associations with other immunotherapies that modulate different aspects of immunity [176].

In a different strategy, Fusciello et al. [177] combined an oncolytic virus (due to its natural adjuvant properties) and cancer cell membranes carrying tumor antigens. They found that viral transduction was significantly increased with the coated virus, implying an uptake mechanism different than that utilized by the naked virus, which requires CAR, representing a significant advantage for transducing CAR-negative cell lines. Additionally, the coated virus was better able to control tumor growth compared to other treatments. The vaccination using coated viruses created a highly specific anticancer immune response, redirecting the immune response against the tumor [177]. Thus, personalized cancer vaccines can represent an alternative approach to target cancer even without determining specific antigens for each patient. We hypothesize that this approach will also be applicable to nonreplicating adenoviral vectors, though this has not yet been shown.

4.5. Association of Antibodies and Viral Structures

The incorporation of antibodies into the viral structure is another interesting option for creating specificity. Despite the obstacles to the use of conventional antibodies (human, murine, and goat), smaller molecules from other species, such as alpacas, can be added to the structure of the Ad capsid without disturbing its synthesis and assembly. For example, van Erp et al. [178] generated a single domain camelid antibody against the human carcinoembryonic antigen present in human colorectal adenocarcinoma cells. They incorporated this molecule into the adenovirus capsid, achieving a more specific tropism for tumor cells and reducing off-target toxicity. Although the strategy was developed to retarget oncolytic viruses, it can also be used to improve nonreplicative adenoviral vectors.

Despite the cited possibilities, the need to re-engineer vectors de novo for each novel target may be an unnecessary and costly effort. Since it is possible to combat different kinds of cancers through similar molecular mechanisms, such as the induction of immunogenic cell death, the development of adaptable platforms may allow the establishment of virus-based therapies in a more scalable and affordable way. Such approaches may, in the future, permit low-effort adaptation of pre-existing therapies to target different cellular markers and treat other tumors.

A viable alternative may be the use of adapter molecules. Bhatia et al. [179] developed an anti-CXCR4 bispecific adapter (sCAR-CXCL12). Chemokine receptor type 4 (CXCR4) is known to be overexpressed in a wide variety of cancers, such as melanoma [180] and breast cancer [181], and it is associated with metastasis and poor overall survival. Bhatia et al. [179] designed a recombinant adapter molecule composed of an ectodomain portion of the human CAR, followed by a 5-peptide linker (GGPGS) and a 6-His tag sequence, fused to the mature human chemokine CXCL12/SDF-1a sequence (CXCR4 ligand). According to the researchers, this bispecific adapter attenuated liver infection in vivo and a promoted a considerable increase in cancer cell infection, as observed in xenograft tumors in mice.

In another interesting work, Schmid et al. [182] achieved, simultaneously, the retargeting of type 5 adenovirus tropism to a specific cancer marker and the reduction of its liver sequestration. Unlike other adapter strategies, they utilized designed ankyrin repeat proteins (DARPins). Similar to antibodies, these proteins can bind to a target with rather

good specificity. Moreover, these molecules can be engineered to target different antigens on the cell surface [183]. Schmid et al. [182] designed an adenovirus-antigen adapter composed of three monomers. Each monomer was made of a retargeting DARPin, a flexible linker, a knob-binding DARPin, and a trimerization motif [182,184]. The last component is responsible for the stability of the complex, allowing the coating of the adenovirus fiber knob and, consequently, impeding virus natural tropism. In addition, according to the researchers and some early works, the removal of CAR and integrin interactions may reduce liver tropism [185,186], an effect also observed when those capsid sites are blocked by DARPin adapters. Furthermore, this protein was able to hide the region responsible for adenovirus–liver interaction without disturbing the adenovirus–integrin interaction. Nonetheless, the researchers developed an adenovirus-binding molecule, named "shield", derived from humanized antihexon scFv, which was designed to bind to hexon proteins, effectively protecting them from neutralizing antibodies [182].

4.6. *Genetic and Chemical Capsid Modifications and Association with Polymers*

Other strategies have emerged that support the retargeting and detargeting of adenoviral vectors. For example, the CGKRK peptide mediates the targeting of tumor cells and tumor neovasculature and has been tested for its ability to retarget PEGylated adenoviral vectors: PEG molecules are conjugated to the surface of the viral vector; then, the peptide is attached via a chemical reaction, resulting in its conjugation to the functional group of PEG [187]. Moreover, Bonsted and colleagues demonstrated that a linker between the poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) and the epidermal growth factor (EGF), commonly overexpressed in tumors, efficiently transduced CAR-deficient cells [188,189]. Additionally, an EGF mimetic peptide linked to the cationic PAMAM (polyamidoamine) dendrimer polymer through a PEG linker has been used to retarget dendrimer-coated Ad vectors; it has been shown to increase transgene expression in target cells compared with the untargeted vector [190].

Kreppel et al. [191] introduced a genetic–chemical concept for vector re- and detargeting. For that, the authors genetically modified the virus in order to present cysteine residues in the capsid, including the fiber HI-loop [191], protein IX [192], and hexon [193]. The cysteine residues were then covalently modified with thiol-reactive coupling moieties, including ligands, shielding polymers, carbohydrates, small molecules, and fluorescent dyes [194]. Kreppel et al. demonstrated that amine-based PEGylation and thiol-based coupling of transferrin to the fiber knob HI-loop successfully retargeted the modified Ad vectors to CAR-deficient cells [191].

These studies highlight the possibility of creating adenoviral vector platforms that need no further genetic modification; thus, a wide variety of target tissues may be explored with the aim of improving specificity and decreasing the neutralizing effects of preexisting antibodies.

5. Conclusions and Future Perspectives

Many features make Ads interesting vehicles for the delivery of foreign antigenic proteins or gene therapy: large cloning capacity, genetic stability, and high in vivo transduction capacity in both dividing and nondividing cells. The natural antiviral immune response can be useful to reprogram the tumor microenvironment from "cold" to "hot" by inducing T-cell-specific immune responses and proinflammatory cytokine expression [195]. The success of therapy depends on several other factors, such as the quality, intensity, specificity, and half-life of immune responses against the tumor. In this scenario, neoantigens have emerged as an attractive target for cancer therapy. Major advances in using the non-self-peptides are the absence of pre-existing central tolerance, potential strong immunogenicity, and lower risk of autoimmunity diseases [196]. We expect that continued refinement of Ad vector design and a deeper understanding of neoantigens will converge to provide an exceptional platform for cancer immunotherapy.

Even so, we point out some limitations for the use of neoantigens in personalized medicine: (1) neoantigens are limited by the diversity of somatic mutations in different tumor types and their individual specificity; (2) the probability that the neoantigens are shared between patients is very low; (3) identification and verification of neoantigens is still time-consuming and expensive [197]. In addition, the construction of adenoviral vectors encoding each neoantigen would be costly and time-consuming; thus, approaches that do not require vector construction may be preferable, including the use of peptides and membrane coatings.

Otherwise, the effectiveness in the use of neoantigens has already been observed in preclinical [198,199] and clinical data [200–202]. In addition, patients with high mutation burden tumors, like melanoma [203,204], non small-cell lung cancer [205], and bladder cancer [206], have had more clinical benefit from checkpoint-blockade therapy than those with lower mutation loads [196]. Moreover, the prediction of peptides binding to MHC molecules and, consequently, the identification of neoepitopes able to stimulate the immune response are emerging as novel approaches that could be associated with adenoviral vectors, reversing part of the tumor-induced immunosuppression.

Recently, D'Alise and collaborators [207] demonstrated the satisfactory benefits of genetic vaccines based on Ads derived from nonhuman great apes (GAd) encoding multiple neoantigens applied in the CT26 murine colon carcinoma model. Both prophylactic and early therapeutic vaccinations elicited strong and effective T-cell responses and controlled tumor growth in mice. The tumor-infiltrating T-cells were diversified in animals treated with GAd and anti-PD1 compared to anti-PD1 alone [207]. The big challenge of neoantigens is the complexity in identifying immunogenic antigens unique to each patient. However, more optimized sequencing platforms and bioinformatics tools are helping to make personalized therapy truly viable. All in all, the data presented here highlight new perspectives of cancer vaccines and gene therapy using modified nonreplicating adenoviruses and different strategies to turn the immune response against the tumor more specific and robust, contributing to local and distant control of tumor progression.

Although viral delivery systems are quite promising strategies in gene therapy, there are some limitations to their clinical application. The major barriers are host immune responses that result in the clearance of vectors, interaction with plasma proteins, liver sequestration, Ad CAR-dependence, and off-target effects [208]. Regarding these issues, a number of genetic manipulations have been exploited to redirect adenovirus binding to different cell surface receptors and, consequently, increase affinity for the target, with lower adverse effects [50].

In this scenario, different strategies using coated viruses have emerged in recent years, and both biological and chemical approaches can be used to coat the virus and improve delivery, especially for the systemic route. Since these strategies involve using cancer cell membranes that can be obtained directly from tumor cell lines, they provide greater biocompatibility with the tumor site and, consequently, specifically target these cells [209]. A growing body of evidence suggests that cancer cell membrane-coated viruses can be delivered by the systemic route, improving the targeting of metastases, with higher retention time, lower immune recognition, and decreased liver sequestration, toxicity, and accumulation in healthy tissues. The induction of immunogenic cell death by nonreplicating Ad vectors is associated with innate immune responses, antigen processing and presentation, and, finally, the activation of the cellular immune response. While few examples currently exist of using membrane-coated adenoviral vectors, we hypothesize that this approach will continue to be studied, including in nonreplicating vectors.

In summary, improvements in vector delivery and targeting will provide an even greater potential for the use of nonreplicating adenoviral vectors in cancer immunotherapy. In particular, we envision vectors adapted to support systemic delivery, achieve tumor specificity, induce tumor cell death and supply specific antigens to guide antitumor immune responses.

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