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Chapter 5

Gene-based Interventions for Cancer Immunotherapy

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Abstract

Immunotherapy of cancer has deservedly gained much attention in the past few years and is likely to continue to advance and become a fundamental cancer treatment. While vaccines, chimeric antigen receptor (CAR) T cells and checkpoint blockade have received the lion's share of the attention, an important direct role for gene transfer as an immunotherapy is emerging. For example, oncolytic viruses induce immunogenic cell death, thus liberating both antigens and the signals that are necessary for the activation of antigen-presenting cells, ensuring stimulation of an adaptive response. In another example, transfer of prodrug converting enzymes, such as the herpes simplex virus-thymidine kinase (HSV-tk) gene or the cytosine deaminase gene, has been shown to promote an immune response, thus functioning as immunotherapies. Alternatively, our own work involves the use of nonreplicating viral vectors for the simultaneous delivery of gene combinations that promote both cell death and an immune response. In fact, our gene transfer approach has been applied as a vaccine, immunotherapy or *in situ* gene therapy, resulting in immunogenic cell death and the induction of a protective immune response. Here, we highlight the development of these approaches both in terms of technical advances and clinical experience.

Keywords: vaccine, CAR-T cell, oncolytic virotherapy, suicide gene, viral vectors

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1. Introduction

The idea that the immune system acts as one of the barriers to the emergence and progression of tumors was conceived more than a 100 years ago [1]. Frank Macfarlane Burnet proposed the concept of "antitumor surveillance," which postulated that the immune system acted as a sentinel that controls and eliminates malignant cells [2]. This hypothesis was much debated and the lack of experimental evidence due to the technological limitations of the time generated a heated debate [3]. However, extensive data presented in the literature have since strengthened and expanded this concept [4–8].

The elaboration of appropriate immune responses must include the detection of the "self" and "nonself" antigens. For this, the immune system must not react to self-antigens and, at the same time, must detect threats to the organism, whether internal or exogenous. Tumors are particularly complex since these unwanted cells arise from the body's own tissues. Thus, upon detection of tumor cells, the immune system must strike a fine balance between activation of effector responses and tolerance. The immune system exploits the tenuous differences between normal cells in homeostasis and intrinsically related tumor cells.

Considering the high rate of mutation in tumors, the newly formed protein variants generate neoepitopes that may serve as targets for the detection and elimination of these aberrant and decontextualized cells by the immune system. These neoantigens can be, for example, the result of mutations caused by dysfunctional chromosomal recombination, such as the Philadelphia chromosome, generating a BCR-ABL gene fusion that did not previously exist in the body. This is a classic example of tumor-specific antigen (TSA), as seen in **Table 1**. Among the solid tumors, melanoma has the highest mutation rate (0.5 to >100 mutations per megabase), which reinforces the hypothesis that it is a highly immunogenic tumor [9]. Another characteristic of tumor cells is that they can express, or overexpress, genes outside the homeostatic context of their microenvironment, such tumor-associated antigens (TAAs) include oncofetal genes (linked to embryogenesis) or tissue markers, such as in melanoma (MAGE) or in breast cancer (HER2) [10]. These neoantigens and deregulated/overexpressed proteins are important targets for immunotherapeutic approaches.

Human tumor	Antigenic protein
Melanoma, esophageal and liver carcinoma	MAGE
Melanoma	Tyrosinase
Breast and ovarian carcinomas	HER2/Neu
Prostate carcinoma	Prostate-specific antigen (PSA)
Head-and-neck carcinoma	Caspase 8
Chronic myelogenous leukemia (CML)	BCR-ABL
Colon carcinoma	Carcinoembryonic antigen (CEA)

Table 1. Examples of TAA and TSA recognized by T cells.

Although antitumor immune responses do occur, tumors often develop elaborate strategies of evasion. This fundamental hallmark of cancer encompasses a wide variety of mechanisms and appears to exploit multiple levels and different cell types in the immune system, acting like a network. These mechanisms include (i) immunoediting, where the selection of variants of nonimmunogenic tumor cells (a phenomenon also known as immunoselection) is due to low expression of immunogenic molecules (like TAA) and/or major histocompatibility complex-I (MHC-I) molecules, and (ii) immuno-subversion, where immune suppressor signals are generated, thus disarming antitumor defenses [5, 7, 11].

These antitumor immune responses rely on innate and adaptive mechanisms. NK (natural killer) cells, part of the innate immune response, recognize MHC-I molecules (through the NKG2D receptor) and eliminate cells that have null or low expression. In addition, danger-associated molecular patterns (DAMPs) and stress-signaling proteins (MICA, MICB, ULBP4) signal an NK attack on cells that have suffered damage and that should be eliminated. Conditions such as irreparable levels of mutations and viral infections naturally trigger this signaling. However, the neoplastic cells can downregulate the expression of these stress markers and MHC-I or may secrete soluble MICA, thus avoiding NK cell attack [12].

Adaptive immunity also undergoes profound changes during tumor progression. Suppressive immune responses include the formation and recruitment of regulatory T lymphocytes (Treg), which, under normal conditions, inhibit the immune system's response to self-antigens [11]. In the tumor context, this mechanism is subverted to suppress antitumor immune responses, generating tolerance to tumor antigens [13–15]. These infiltrating Tregs contribute to the establishment of a tumor microenvironment abundant in immunosuppressive factors (IL-10, TGF-β, Arg1 and IDO) that influence many different cellular types, including the inhibition of effector T lymphocytes (Teff), generation of myeloid-derived suppressor cells (MDSC) and impairment of the proper function of dendritic cells (DCs) for presenting antigens [7].

The immunosuppressive tumor microenvironment also influences the immune checkpoint status, promoting the expression of inhibitory checkpoint molecules (CTLA-4, PD-1, IDO, LAG3, TIM3 and KIR) to the detriment of stimulatory checkpoint molecules (OX40, CD27, CD28, CD40, CD122 and ICOS) [16]. On the other hand, understanding this phenomenon brings interesting perspectives for immunotherapies as discussed below.

2. Immune interventions promoting active responses against tumor cells

Therapeutic strategies that target immune activation have shown significantly increased survival and quality of life for cancer patients [17]. Cancer immunotherapy comprises a variety of treatment approaches and combinations, incorporating the specificity of the adaptive immune response (T cells and antibodies) as well as the diverse and potent cytotoxic weaponry of both adaptive and innate immunity [18]. In this section, we provide an overview of key immunotherapeutic approaches.

Some of these strategies involve the application of soluble antibody molecules that specifically recognize and bind TAAs, resulting in blocked receptor signaling and/or passive immunotherapy. In particular, targeting tumor cells by engaging surface antigens differentially expressed in cancers has been widely used. For example, rituximab targets CD20 in non-Hodgkin B cell lymphoma. At least nine monoclonal antibodies (mAbs) targeting six TAAs (HER2/Neu, EGFR, VEGF, CD20, CD52 and CD33) are approved for the treatment of solid and hematological malignancies [19].

Approved by the Food and Drug Administration (FDA) in 2011, ipilimumab is a mAb against cytotoxic T lymphocyte–associated protein 4 (CTLA-4), a negative checkpoint of T cell function. Thus, checkpoint blockade with ipilimumab releases the brakes of the immune system, promoting T cells to combat cancer cells, and has already benefited thousands of patients with advanced melanoma, a disease that typically kills in less than a year [20]. Additional targets of immune checkpoint therapy include programmed cell death protein 1 (PD1) and its ligand PD-L1, which are even more effective and have fewer side effects as compared to anti-CTLA4 [21]. Moreover, checkpoint inhibitors may be used in combination with each other or with other therapies resulting in the induction of sustained antitumor responses in a wide variety of tumors [22–25]. Checkpoint blockade has undoubtedly been one of the most impressive advancements in cancer therapeutics in recent years, prolonging and saving the lives of many cancer patients. Even so, this approach does not directly induce a *de novo* immune response but releases experienced T cells from inhibitory signaling.

Vaccines are strategies to activate effector immune cells upon stimulation with tumor antigens, promoting the patient's own immune system to mount an immune response against neoplastic cells. Numerous vaccine approaches have been attempted and share the goal of providing effective target antigens while reverting, perhaps, the immunosuppressive tumor microenvironment and activating the ability of DCs to present these antigens. One example is GVAX (Cell Genesys, Inc., South San Francisco, CA), a polyvalent vaccine derived from a cultured cancer cell line expressing a plurality of shared tumor antigens. In addition, the cells have been genetically modified to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), an immune-modulatory cytokine that can activate antigen-presenting cells (APCs) locally at the vaccine site. Indeed, autologous and/or allogeneic GM-CSF-secreting tumor cell vaccines have demonstrated evidence of immunologic responses in patients with various types of cancers, for example, chronic myeloid leukemia [26], melanoma [27], pancreatic adenocarcinoma [28] and prostate cancer [29].

Oncolytic virotherapy (OV) is a novel form of cancer therapy that employs native or engineered viruses that selectively replicate in and kill cancer cells. OVs act as immunotherapies, promoting antitumor responses due to the viral infection of tumor cells and their acute lysis. An example of this therapy is an intralesional injection with talimogene laherparepvec (Imlygic, T-VEC, Amgen, Thousand Oaks, CA), a genetically engineered oncolytic HSV (herpes simplex virus), with mutations in infectious cell proteins (ICPs) 34.5 and 47, and expressing US11 and GM-CSF [30].

Alternatively, the patient's own T cells or NK cells may be used as a therapeutic agent. Such adoptive cell therapy (ACT) involves the recovery and *ex vivo* expansion of the patient's cells, providing the opportunity for selection and activation of tumor-specific populations, before

they are infused in the patient [31]. One of the most advanced ACTs in clinical use is called CAR (chimeric antigen receptor) T cell therapy, which involves genetic modification of the patient's T cells to enhance their ability to recognize and attack cancer cells [32]. CAR-T cells have been engineered to express multiple CARs that recognize several tumor antigens. This technology has been successfully applied in clinical trials for hematological malignancies, with durable and complete remission in acute lymphoblastic leukemia [33], chronic lymphocytic leukemia [34] and B-cell lymphomas [35]. Another interesting application is the introduction of CARs targeting negative regulatory receptors, such as PD-1, resulting in reversal of immunosuppression in the tumor [36].

While cancer immunotherapies continue to evolve, the recurring role for gene transfer as a fundamental component of many of these approaches is quite evident. Here, we explore several immunotherapy approaches that rely on some aspects of gene transfer, highlighting both clinical and technological advances, especially as related to virotherapy, suicide genes, vaccines and CAR-T cells.

3. Cancer vaccines

Genetic instability intrinsic to cancer generates innumerable missense mutations in tumor cells and thus generates specific targets for T cell immunity [37]. Since these neoantigens are not expressed in normal somatic cells, they are inviting targets for the development of cancer vaccines and rational combinations of immunotherapies [38].

Although the term vaccine initially referred to the use of prophylactic immunizations for bacterial or viral infections, there are vaccines for therapeutic purposes, especially when we refer to cancer. This strategy has been gaining prominence lately as it offers the opportunity for a lasting effector response and with far fewer side effects than established traditional treatments, such as chemotherapy. In general terms, cancer vaccines seek to restore the ability of the immune system to recognize and eliminate neoplastic cells. In addition, the possibility of generating memory T cells favors long-lasting protective effects, including the prevention of metastasis after primary remission, which would greatly increase the survival and quality of life of these patients.

One of the earliest reports of cancer immunotherapy was conducted by William B. Coley. After observing that established tumors associated with fever or infection generally had higher rates of spontaneous regression, Streptococcus (Coley's toxin) was injected into an inoperable bone tumor. Despite generating data with difficult interpretation, it sparked a debate and numerous other fronts of investigation [39]. Corroborating this hypothesis, Lamm et al. demonstrated that Bacillus Calmette-Guerin (BCG) could be used to activate the immune system and thus enable the treatment of bladder cancer. This therapy, approved by the FDA, is still in clinical use [40].

3.1. Improving vaccine efficacy

In both of the pioneering works described above, bacterial components having immunostimulatory properties were used. It is now clear that the formulation of vaccines should include

adjuvants, important components for immunomodulatory actions or acting as delivery systems for vaccine antigens [41–43]. The adjuvants' property of modulating the immune system is in part due to their interaction with the receptors of pathogen-associated molecular patterns (PAMPs). Toll-like receptor (TLR) and the Nod-like (nucleotide oligomerization domain) receptor families, for example, mediate the cellular response to PAMPs [44, 45]. Different classes of TLRs each recognize a specific molecular pattern. Briefly, TLRs 1, 2, 4, 5 and 6 recognize molecular patterns associated with bacteria. On the other hand, TLRs 3 and 7 are specialized in the recognition of molecular patterns associated with viral dsRNA and ssRNA, respectively. While TLRs 8, 9 and 13 recognize patterns of viruses and bacteria concomitantly, associated with ssRNA, DNA CpG patterns and ribosomal RNA sequences, respectively [46, 47]. The possibility of synergy when different innate receptors are stimulated may further enhance the adaptive immune response [48].

Several vaccine strategies may be employed for delivery of tumor antigens, adjuvants and modulators of the immune response (**Table 2**). Each strategy has its strengths and weaknesses. Even when meticulously planned, the actual response seen in clinical trials is often unpredictable. The vaccine regimen, number of doses, dosage, route of administration and adjuvant employed are variables that directly influence the type and intensity of the immune response generated. Another important point to be weighed is the mechanism of action, including (i) passive therapies based on the transfer of molecules (such as antibody or cytokine therapies) or mature immune effector cells for example transfer of adoptive T cells, or even CAR-T cell–based therapy; or, (ii) active therapies including classical therapeutic vaccines and those based on DCs to establish effector immune responses against tumors.

Protein-based immunotherapy combines peptides and/or proteins, aiming to activate antitumor immune responses. This strategy has been particularly effective in preventing oncogenic virus infection, as has been seen with Gardasil and Cervarix, which block HPV-associated cervical cancer [49]. The immune responses to structural proteins or viral oncoproteins are likely to be more effective since these antigens are foreign in the body. However, cancer-associated proteins or epitopes, being self-antigens, are naturally less immunogenic and typically associated with immune tolerance; consequently, they are less effective in eliciting immune responses in preclinical cancer models. In this way, delivery systems involving peptides, proteins and DNA/RNA vaccines, although classically used, may be poorly immunogenic and require appropriate pairing with adjuvants [50–52].

On the other hand, delivery systems based on viral vectors can be used for this purpose and may offer greater immunogenicity. Considering that many viral vectors come from pathogenic viruses such as lentivirus, retroviruses and adenoviruses, there is already a line of defense against these "intruders" that can be raised during immunotherapy. This strategy has inherent advantages, such as the possibility of activating innate immune responses due to a variety of viral molecular patterns that are agonists of TLRs, attracting and helping to mature cells of the adaptive immune response. As for the safety of these vectors, genetic engineering techniques allow the removal of specific genes related to pathogenicity, making them innocuous and safe for human use [53, 54]. In the last few years, several virus-driven therapies have been approved for human use, showing substantial progress in the field of gene therapy. Such approaches include Glybera for lipoprotein lipase deficiency and the oncolytic

Type	Generic mechanism	Clinical trials[a]	FDA approved
Protein based			
Peptides/proteins	Provide epitopes for specific antitumor immune responses.	446/278	Gardasil
Cytokine therapy	Modulate positively antitumor immunity.	721	Proleukin (IL2r)
Antibody therapy	Selectively target dysfunctional or overexpressed proteins in tumors.	4187	Rituximab, bevacizumab, ipilimumab, pembrolizumab
Gene based			
DNA/RNA vaccines	Provide epitopes for select antitumor immune responses.	142/80	–
Recombinant virus based			
Adenovirus	Gene transfer, including TK, CD and cytokines, among other transgenes.	193	–
Oncolytic virus	Selective infection in tumors promoting cell death.	74	Imlygic
Cell based			
Tumor cells	Provide wide range of epitopes for select antitumor immune responses.	78	–
Dendritic cells	Provide mature, activated and antigen-loaded dendritic cells for the correct antigen presentation, and consequent generation of effector T cells against the tumors.	574	Sipuleucel-T
Transfer of adoptive T cells	To provide T lymphocytes with lithic capacity directed at tumor cells.	77	–
CAR-T	T lymphocytes engineered *in vitro* that recognizes proteins/tumor epitopes, being endowed with lytic capacity independent of costimulatory molecules.	342	Kymriah

[a]clinicaltrials.gov, search performed April, 2018.

Table 2. Type of gene transfer used in vaccines and immunotherapy against cancer.

virotherapy Imlygic [55, 56], CAR-T cell immunotherapies Kymriah and Yescarta [57], as well as Strimvelis for the treatment of ADA-SCID (severe combined immunodeficiency due to adenosine deaminase deficiency) [58] and Luxturna for the treatment of Leber's congenital amaurosis [59].

The efficiency of immunotherapies may be increased by applying combinations of different strategies. The combination of antibody therapy, cytokine therapy and checkpoint blockade with other immunotherapeutic strategies has been shown to increase antitumor activity [60–62]. Antibody therapy often targets tumor antigens and/or tumor-promoting proteins. Some antibodies act as blockers of the function of their targets, while others may act as agonists. Additionally, the binding of these antibodies to their targets may direct opsonization or

complement-mediated lysis and thereby contribute to the elimination of tumor cells. Another aspect of passive immune therapies is the use of recombinant cytokines, such as IL-2, IL-12 and interferon-α, β and γ [63]. Although both strategies can modulate the immune system to bring improvements, their action is temporary and can only be palliative, requiring successive doses and may provoke serious adverse effects [64, 65]. Checkpoint blockade has been gaining prominence recently and also encompasses the use of monoclonal antibody inhibitors of negative modulators of immune function, such as anti-PD-1, PDL1 and CTLA4 [66–68].

3.2. Modified dendritic cells as therapeutic vaccines

The presentation of antigens is a crucial event in the genesis of adaptive immune responses. Antigen-presenting cells (APCs) capture proteins in peripheral tissues, process them by proteolytic digestion and, after migrating to secondary lymphoid organs, present them to T lymphocytes in the context of class I or II MHC molecules [69]. In addition to the MHC molecules (HLA in humans), a number of costimulators (such as CD80, CD86, CD40, CD83 and CD14) are also required, important for the complementation of the biochemical signals necessary for the activation of T lymphocytes upon recognition of the presented antigens [70–72]. The maturation of cytotoxic T lymphocytes is central to the generation of adaptive immunity and, in turn, is one of the major antitumor defenses.

Autologous dendritic cell vaccines can be prepared from the patient's peripheral blood, with isolation of $CD14^+$ cells and *in vitro* treatment with GM-CSF and IL-4 for differentiation and maturation of monocyte-derived DCs (Mo-DCs). Next, different techniques can be used to "load" the tumor antigens into the DCs, such as peptides, proteins, DNA or RNA transfection, exosomes or exposure to tumor cell lysates [73, 74]. In addition to the changes that occur in the tumor microenvironment, the tumor is also capable of inducing systemic changes in the host's immune system, so that the monocytes from cancer patients may result in DCs with altered phenotype and cytokine production, negatively impacting immunotherapy [15]. Thus, immunotherapy with allogeneic DCs represents an interesting alternative. In addition to offering greater availability of DCs (since healthy donors have higher monocyte counts), tissue rejection by antigenic determinants (HLA) may function as an adjuvant.

Barbuto et al. used an interesting strategy for the construction of DC-based therapeutic vaccines for cancer. Healthy donor monocytes are differentiated and matured *ex vivo* and are subsequently fused to tumor cells by electrical shock, resulting in a hybrid cell. These hybrids are gamma irradiated, to prevent replication, and then administered back to the patient, seeking the generation of immune responses against neoplasms. Although the hybrids were shown to offer limited improvement of mortality rates, longer survival of the treated patients was achieved [75, 76]. Another phase I study in melanoma patients employed immunotherapy using plasmacytoid and myeloid DCs (pDC and mDC, respectively). The results were promising and indicated a survival time of more than 2 years in most of their patients [77, 78].

Currently, more than 500 clinical trials using dendritic cells are being conducted for the treatment of various forms of cancer in different countries. Most of these (324) are in the US, followed by the European Union (120) and China (72) [79]. Although results are very heterogeneous, there is a consensus that the use of these therapies in humans does not present risks or serious side effects.

Sipuleucel-T (Provenge), a dendritic cell-based vaccine for the treatment of metastatic castration-resistant prostate cancer, is the only example approved for use in humans. Its manufacture is done in a personalized manner, which involves the extraction of the patient's peripheral blood mononuclear cells (PBMCs) by leukapheresis, transport of the cells to Dendreon's facility (New Jersey, USA) for in vitro culture, maturation of DCs and loading with PA2024 (hybrid protein of GM-CSF and prostate-specific prostatic acid phosphatase, PAP) before returning the cells to the hospital where they will be administered to the patient [80].

Three phase 3 clinical trials supported the approval of sipuleucel-T by the FDA [81–83]. These studies have demonstrated that sipuleucel-T extended the survival of treated patients by 4.1 months when compared to the control group that received cells processed in a manner similar to sipuleucel-T, however, without activation due to the absence of the recombinant protein. Although this gain in survival seems promising, none of these studies showed significant increase in time to disease progression [84]. However, no side effects were observed in most cases, and T-lymphocyte proliferation was also detected, factors contributing to FDA approval [84]. In practice, the logistics of sending temperature- and time-sensitive material from widely distributed health care institutions to and from a single processing center made this immunotherapeutic strategy cumbersome and relatively expensive, since the total cost of treatment with sipuleucel-T has been reported to be $93,000 to $140,000.00 [80, 85]. Despite the prolonged survival and increased quality of life, this therapeutic option was not sustained and was discontinued.

4. Suicide gene therapy

In cancer gene therapy, different approaches can be used to kill tumor cells. Suicide gene therapy (also called gene-directed enzyme prodrug therapy) is one example where a viral or bacterial gene is introduced in the cancer cell such that it can convert a nontoxic prodrug into its lethal form. The most famous system used in this strategy is herpes simplex virus thymidine kinase gene (HSV-tk) and ganciclovir (GCV) as the prodrug. Expression of the HSV-tk gene leads to production of the enzyme that turns GCV into GCV monophosphate. After this first conversion, cellular kinases metabolize GCV monophosphate into GCV triphosphate, which is an analogue of deoxyguanosine triphosphate. GCV triphosphate causes tumor cell death upon its incorporation into DNA and consequent inhibition of DNA replication [86]. Another example of a suicide gene is the cytosine deaminase gene (CD) of *Escherichia coli* that catalyzes the hydrolytic deamination of cytosine into uracil, converting the nontoxic antifungal agent 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU). This process causes cell death by three main pathways: thymidylate synthase inhibition, formation of (5-FU) RNA and of (5-FU) DNA complexes [86]. More recent systems were developed, including an engineered version of human thymidylate kinase (TMPK) and the prodrug azidothymidine (AZT), which was first tested in leukemia model *in vitro* and *in vivo*. Native TMPK catalyzes AZT into AZT monophosphate, the toxic compound, only very slowly, so the engineering of TMPK allows it to act more robustly [87, 88]. In another example, the iCas9 system consists of inducible expression of the caspase-9 gene and administration of the small molecule chemical inducer of dimerization (CID) that leads to caspase-9 dimerization, thus promoting apoptosis [86].

One of the advantages of the suicide gene approach is the bystander effect that consists of a functional effect that may be seen even when only a small percent of cells has been transduced, and thus, tumor regression can occur. The most accepted hypotheses for this phenomenon of killing nontransduced tumor cells are passive diffusion of the drug, passage of the drug through gap junctions and release of soluble factors, forming a local bystander effect [89]. A different approach that relies on the bystander effect involves the use of mesenchymal stem cells (MSCs) to deliver drugs or vectors. The advantage in this case is that HSV-tk–modified MSCs could be effectively delivered to the area of interest and GCV could then be safely administrated systemically. HSV-tk–bearing MSCs home to and infiltrate the tumor region. Consequently, only tumor cells will be affected, while adjacent areas should remain unharmed [90].

Alternatively, the bystander effect may be a consequence of an immune response initiated by suicide gene therapy *in vivo*, also known as a distant bystander effect. Several articles in the literature have demonstrated a relationship between HSV-tk and immune response. Also called gene-mediated cytotoxic immunotherapy, treatment with HSV-tk promotes innate immune stimulation and infiltration of T cells in tumors [89]. In a clinical trial treating prostate cancer, Ayala and collaborators used an adenoviral vector encoding HSV-tk. In addition to increased apoptosis and decreased microvessel density, they found circulating and activated CD8+ cells and increased IL-12, an important mediator of immune response to tumor cells and viral infection. They also found intratumor CD8+ cells, suggesting the occurrence of both local and systemic responses [91]. Combining suicide and immune gene therapy in an aggressive melanoma model, together HSV-tk and GM-CSF induced a meaningful systemic immune response that was stronger as compared to GM-CSF alone [92]. The induction of an immune response upon CD/5-FC may be less well known [93] but has also been reported [94, 95]. Adenoviral delivery of HSV-tk was tested in a phase III trial, showing increased time to death in patients with high-grade glioma, but it did not increase overall survival [96]; perhaps combining suicide gene therapy with an additional immunotherapy approach could improve response. For example, a current trial is testing the combination of HSV-tk with FMS-like tyrosine kinase 3 ligand (FLT3L) carried by adenoviral vectors in order to promote both tumor cell death and DC activity [97].

Applied as a safety mechanism, HSV-tk is also used to control CAR-T cells. As described in more detail below, the successful clinical experience of engineered CAR-T cells is also associated with serious adverse events where the massive cell killing results in tumor lysis syndrome, an extreme elevation of plasma IL-6 concentrations that can lead to hypotension and respiratory distress in severe cases [98]. Accordingly, suicide gene therapy can be used to kill the CAR-T cells and thus stop the cytokine release syndrome [99]. In a myeloid leukemia model, Casucci and collaborators associated HSV-tk/GCV with CAR-T cells targeting the CD44v6 receptor and compared this approach with the use of the nonimmunogenic suicide gene iCas9 in an attempt to avoid an unwanted immune response, revealing that the second approach was more effective in containing the cytokine release syndrome [100]. At least three clinical trials utilizing iCas9 to control cell fate upon adoptive T cell transfer have been initiated for the treatment of leukemia and lymphoma [79, 101].

In summary, suicide gene therapy is an approach that involves death mechanisms and immunotherapy. The strategy is still evolving from the initial trials and may be an interesting option against cancer and for the improved safety of CAR-T cell therapy.

5. Turning gene therapy into immunotherapy: adenovirus-carrying ARF and interferon-beta

Our own research has focused on the use of nonreplicating viral vectors for the transfer of tumor suppressor genes in combination with an immune-modulating gene (**Figure 1**). The goal is to induce both cell death and an immune response, thus overcoming the immunosuppressive tumor microenvironment and initiating the cancer immunity cycle. To this end, we have developed an improved vector system that promotes cooperation between gene function and vector performance.

We have constructed a series of viral vectors where transgene expression is controlled by the tumor suppressor p53, a powerful transcriptional regulator [54, 102, 103]. Moreover, placing the p53 cDNA under the control of the p53-responsive promoter (PGTxβ, or simply PG)

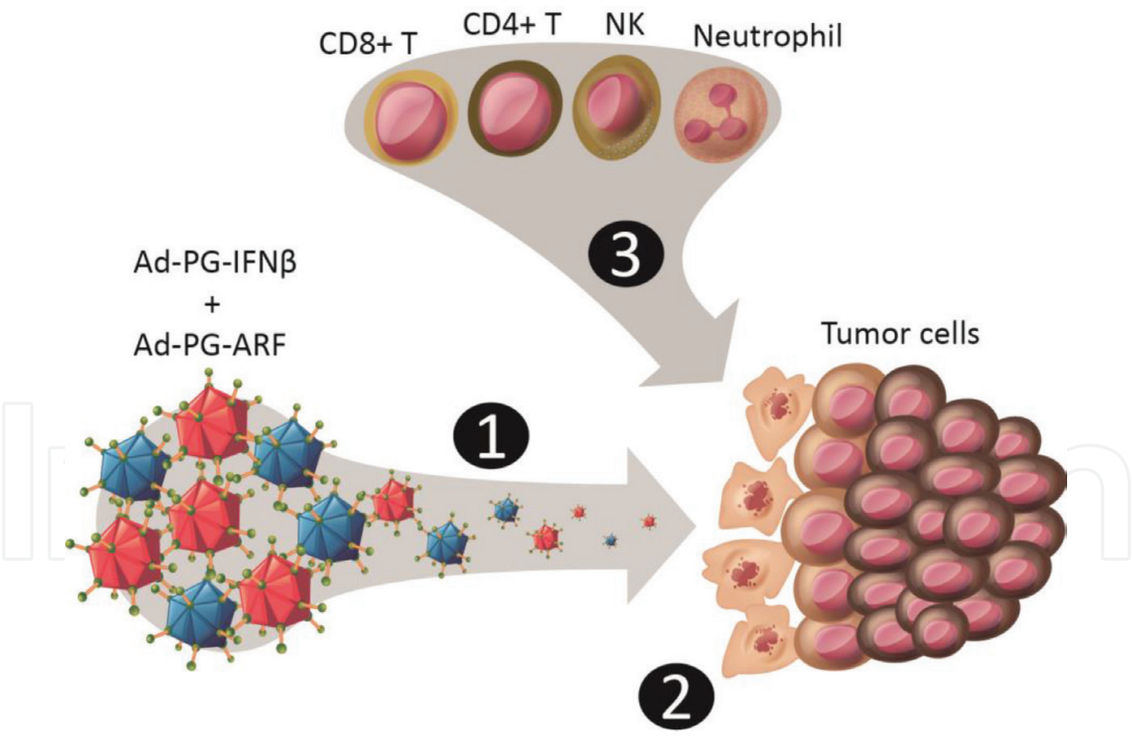


Figure 1. Schematic representation of our immunotherapy approach. (1) The adenoviral vectors encode either interferon-β (IFNβ) or p19ARF (alternate reading frame, p19ARF in mice and p14ARF in humans) where expression of the cDNA is controlled by a p53 responsive promoter, termed PG. (2) The combination of IFNβ + ARF induces tumor cell death by necroptosis and is associated with the release of immunogenic factors (such as HMGB1, ATP and calreticulin). (3) Immune cells are recruited and activated to attack the tumor.

establishes an autoregulatory, positive feedback mechanism that was shown to outperform vectors employing a constitutive promoter to express p53. That is, gene expression and cell killing *in vitro* and *in vivo* were superior when using our modified vectors to express p53 [104–106]. We have also looked to p19ARF (alternate reading frame, p19ARF in mice and p14ARF in humans), a functional partner of p53, to serve as the death-promoting factor in our approach and have observed that it is effectively expressed from our p53-responsive vectors in tumor cells that harbor wild type p53, resulting in activation of p53-mediated cell killing *in vitro* and *in vivo* [107]. Admittedly, cell killing mediated by the p53/ARF pathway alone has a limited, but recognized, role in promoting an antitumor immune response [108].

In order to activate the immune response against the tumor, we have added interferon-β (IFNβ) to our therapeutic approach since it is a central player in innate and adaptive immunity [109]. Indeed, the combination of p19Arf and IFNβ is better able to induce melanoma cell death both *in vitro* and *in vivo* [110, 111]. Strikingly, the mechanism of cell death involves necroptosis with liberation of the classic markers of immunogenic cell death [111]. In a mouse model of melanoma, we have confirmed the induction of an antitumor immune response in vaccine and immunotherapy settings, with critical involvement of NK cells, CD4+ and CD8+ T cells [112]. In a mouse model of lung carcinoma, we have shown that *in situ* gene therapy can bring about an antitumor immune response with critical involvement of neutrophils [113]. Together these studies show that our gene transfer approach is an effective immunotherapy [114, 115]. The results to date are promising and research will continue to evolve, with critical development using clinically relevant models, such as testing with patient-derived tumor samples as well as alternative animal models, including canines [116].

6. Oncolytic virotherapy

In 1892, viruses were first noted by humans and it took only a few years for researchers to raise the possibility that some viral infections may interfere in the clinical outcomes of some patients with different types of cancers. In 1904, a transitory spontaneous remission of acute leukemia in a patient after infection with influenza was reported, prompting the observation of additional occurrences of this type and paving the way for the concept of virotherapy [117]. One of the first reports of viruses being deliberately applied as a therapeutic approach for cancer dates back to 1949, when Herman A. Hoster and colleagues evaluated the clinical outcome of 21 Hodgkin's disease patients after intentional exposure to Hepatitis B virus [118]. Some years after that, Newman and Southam evaluated the use of several different viruses (vaccinia, mumps, West Nile, dengue, among others) for the treatment of advanced cancer in 57 patients, though no remarkable clinical outcome was observed [119].

Concomitant with the expansion of knowledge in the field of virology, additional protocols describing novel attempts to establish cancer virotherapy were reported, including the use of an array of different virus species, such as adenovirus, Coxsackie, and Epstein-Barr. Despite the new investigations in the 1970s, the threshold of "transitory response" could not be surpassed due to adverse events, such as neurotoxicity, possibly associated with technological limits related to the handling of viruses, for example, the lack of genetic engineering tools needed for the development and testing of more effective and safer versions [120].

With the advances in molecular and cellular biology as well as animal models for cancer research, the perspective of taking oncolytic virotherapy (OV) from bench to the bedside became feasible. For example, a report in 1991 described the construction of a modified herpes simplex virus (HSV-1), which was thymidine kinase-negative and attenuated for neurotoxicity [121]; thus, a critical step was taken to advance the technology of OV.

By definition, OV encompasses native or genetically engineered viruses whose replication is restricted to tumor cells. As per the immunotherapy trend, OV is increasingly gaining attention due to its performance in clinical trials where it is used to treat several types of cancers. With the 2015 approval of Imlygic (talimogene laherparepvec, OncoVex, T-VEC, an HSV-based oncolytic virus) by the FDA and the EMA (European Medicines Agency) for the treatment of unresectable melanoma, the principle of taking advantage of viral replication in order to treat cancer is now an established therapeutic approach.

6.1. Targeting and mechanism of OV

Even in the absence of tools to genetically modify viruses in order to make them safer, in the 1950s, Alice Moore observed that it was possible to generate virus strains with higher oncolytic capacity and more tumor specificity through adaptation. In particular, the oncolytic features of Russian encephalitis virus were enhanced after 20–30 passages in the Sarcoma 180 cell line as compared to the original strain, leading to the idea that the tumor cells could exert an evolutionary pressure upon the virus, favoring those particles adapted to replicate in the tumor [122].

After the development of techniques for the manipulation of DNA, these tools were used to break down the barriers for the development of virotherapy. Thus, undesirable virulence could be mitigated by eliminating key genes from the viral genome, generating attenuated viruses. The viral genome often codes important proteins that regulate its replication in post-mitotic cells. For example, the thymidine kinase (TK) gene is associated with DNA synthesis and cell cycle progression [123]. Taking advantage of this information, Martuza and collaborators showed that HSV lacking the gene coding for TK could replicate in dividing cells, but replication was hampered in quiescent cells, in line with the need for selective replication in tumor cells. In an animal model of glioma, locally administrated mutant HSV led to inhibition of tumor growth and showed decreased neurotoxicity [121]. Alternatively, the viral life cycle may be guided by cellular or virus-encoded microRNAs that alter the level of expression of cell-specific proteins [124].

In addition to the aforementioned approaches, tumor selectivity may be achieved by directing the interactions between the virus particle and the target cell. The retargeting of the viral particles can be achieved in different ways, such as the genetic modification of viral proteins so that they gain specificity for a particular cell surface protein. Alternatively, the use of bispecific adapters mediates the interaction of native capsid proteins with a specific cellular receptor. The virus may also be detargeted, that is, modified so that it no longer interacts with nontumor cells [125].

Besides the transductional targeting, the tropism can be also altered at the transcriptional level by using a tissue-specific promoter to regulate the expression of genes critical for viral

replication. As an example, in order to produce adenovirus whose replication is restricted to prostate cancer cells, expression of the *E1A* adenoviral gene (essential for regulating adenoviral replication) was placed under the control of the prostate-specific antigen (PSA) promoter, leading to an adenovirus that is only able to replicate in prostate cells [126].

Viruses themselves are entities capable of subverting the cell replication machinery and making a favorable environment for their own replication, which occasionally leads to cell death by lysis when the new viral particles are released and the infection cycle continues, increasing the initial quantity of viral particles that is then only limited by the decreased number of target cell as well as by the direct action of the immune system through an antiviral response. In addition to lysis due to viral replication, some viruses can produce proteins that trigger molecular pathways that lead to cell death, as is the case for adenovirus, whose E3-11.6 K transcript is found to be important for the lysis of infected cells [127, 128]. However, more recently, it was found that the immune system, concomitant to the intrinsic effect of oncolytic infection, plays an important role.

After infection, more precisely after cell lysis, the release of intracellular content participates in the activation of both innate and adaptive immune responses against tumor- and virus-associated antigens, potentially reverting the intrinsic immune tolerance of the tumor microenvironment [129]. After rupture of the cellular membrane by the virions, the following release of PAMPs and DAMPs induces the activation of type I interferon, Toll-like receptor–mediated molecular pathways and the production of cytokines, which culminate in the recruitment and activation of antigen-presenting cells (APCs) and the subsequent establishment of a memory immune response [130].

6.2. Oncolytic virotherapy makes its mark: oncolytics with regulatory approval for the treatment of cancer

In 2005, Oncorine (H101, Onyx-015), an adenovirus-based oncolytic developed by Shanghai Sunway Biotech, was approved by State Food and Drug Administration, China (SFDA), for the treatment of head and neck squamous cell carcinoma [131]. Oncorine is a modified adenovirus whose E1B and E3 genes are deleted, leading to a virus that, it was originally thought, should only replicate in cells that lack p53 activity, mainly tumor cells [132, 133], though other mechanisms have been proposed for Oncorine's tumor selectivity [134]. Its precursor, Onyx-15, showed good performance in clinical trials, especially when combined with additional therapeutic approaches, and was well tolerated and safe [135], with no therapy-associated severe adverse events when administered intratumorally in gliomas [136]. In addition to its safety profile, Onyx-15 administration may be associated with some clinical improvement for patients with metastatic colorectal cancer who failed the first-line therapy [137] and those with hepatobiliary tumors not eligible for surgical resection [138].

In 2015, the FDA and the EMA approved an OV based on a modified herpes simplex virus (HSV-1) for the treatment of melanoma. Imlygic (OncoVex, T-VEC, talimogene laherparepvec) expresses granulocyte-macrophage colony-stimulating factor (GM-CSF), while viral genes ICP34.5 and ICP47 were deleted, modifications that conferred better replication in tumor cells

and stimulation of an antitumor immune response [30]. After showing safety and antitumor activity in experimental models [30], Imlygic was then administered in a phase I clinical trial, in patients with cutaneous or subcutaneous metastases from refractory head and neck carcinoma, melanoma, breast and gastrointestinal adenocarcinoma, being well tolerated and provoking only mild adverse events (local erythema and fever) [139]. Encouraged by these results, efficacy was assessed in a phase II clinical trial carried out with 50 stages III and IV melanoma patients. In this study, mild adverse events were observed and there was a 26% Response Evaluation Criteria in Solid Tumors (RECIST) response rate, including 8 complete and 5 partial responses [140]. Based on these positive results, an open-label phase III study was carried out where therapy with Imlygic was compared to treatment with GM-CSF, revealing high tolerance to the treatment, and a higher durable response rate (DRR) and also overall survival compared to the GM-CSF treatment, results that culminated in the first FDA and EMA approval of an OV [141].

7. CAR-T cells

An emerging and exciting subject in cellular therapies relies on the engineering of cytotoxic T cells and natural killer cells so they can recognize specific antigens on the cell membrane and induce cell death without reliance on MHC or costimulator expression. Even though infiltrating T cells may recognize tumor antigens, they may be unable to induce a cytotoxic response due to a strong inhibitory microenvironment [142]. The modification of patients' T cells to express a chimeric antigen receptor (CAR) creates the opportunity to induce a strong cytotoxic response against the tumor even in the face of negative signals [143].

Transmembrane CAR receptors have two main functions: the first is to recognize a specific antigen present only in the membrane of tumor cells. The second is to induce signal transduction independently of other costimulatory signals, culminating in the release of cytotoxic signals and T cell proliferation [144]. Physiologically, the activation of a cytotoxic T cell is mediated by a T cell receptor (TCR) in an MHC-dependent context. Though this antigen-receptor interaction is insufficient to bring about cell killing, it is imperative that other transmembrane receptors interact, authorizing T cells to exert their cytotoxic function. Moreover, the tumor has several mechanisms to evade T cell responses, from losing the MHC complex to expressing inhibitory molecules that induce T cell exhaustion and anergy. Therefore, modifying the TCR so they do not depend upon other authorizing signals has proven an exciting strategy [142].

Structurally, a CAR has an extracellular component responsible for recognizing the antigen of interest, comprised of a single-chain variable fragment (scFv), followed by a spacer region whose length may vary, a transmembrane region (TM), and an intracellular domain composed of one or more signaling components associated with T cell activation. The first generation included, on the intracellular domain, the ζ-chain, a portion of the T cell receptor responsible for its activity. Improved understanding of the complementary signals needed for activation lead to the development of second-generation CARs, which include a CD28 costimulatory

domain, thus ensuring full activation of the T cell. The third generation included other transduction signaling domains, preferentially originating from transmembrane proteins derived from the TNF superfamily, such as CD27, 4-1BB and OX40. All of them can transduce signals resulting in survival, proliferation and maintenance of T cells. The fourth generation uses a vector to deliver, in addition to CAR, cytokine genes, such as IL-2 or IL-12, whose expression changes the tumor microenvironment in favor of T cell activity [144].

The first insight into the development of a chimeric transmembrane receptor that could activate cytotoxic T cells came in 1989 by Gross and colleagues. And in 2017, the FDA approved the first two CAR-T cell therapies in rapid succession. These CARs target CD19, a molecule expressed only in B-lymphocytes, an approach shown to be a powerful second-line treatment against B cell acute lymphoblastic leukemia (B-ALL) (Kymriah—tisagenlecleucel, August 2017 [145]) and certain B cell lymphomas (Yescarta—axicabtagene ciloleucel, September 2017 [146]). While both present a scFv against CD19, Kymriah uses the 4-1BB whereas Yescarta uses CD28 as costimulatory domains. The success in clinical trials ranged from 70 to 94%, making these treatments a breakthrough in gene and immunotherapy [144]. However, there are cytotoxic effects that in some cases can be intense, caused by the killing of large numbers of cancer cells that release cytokines and waste products, leading to harmful consequences in the patients. Thus, much more is needed to understand and manage the side effects of these new and promising therapies, such as the inclusion of a suicide gene to eliminate overactive CAR-T cells [147].

Despite the incredible potential of this therapeutic strategy, CAR-T cells have some limitations that prevent their effective use in the fight against a wide range of tumors. Among them, the most troubling is the lack of a perfect antigen present only in tumor cells but not in other tissues. Tandem CAR and inhibitory chimeric antigen receptors (iCAR) are some of the strategies with the greatest potential to overcome this barrier. Tandem CAR consists of two chimeric receptors designed to provide costimulatory signals in response to the recognition of two different antigens [144]. Only after the recognition of both signals are the tandem CAR cells activated. On the other hand, iCAR aims to inhibit T cell activity as soon as the second specific antigen is recognized [144].This second antigen does not exist in tumor cells, so when the iCAR-T cells find it, they are inhibited and leave nontumor cells unscathed.

In some studies, the inhibitory molecules used in the construction of iCAR-T cells are derived from the intracellular domains of proteins often expressed by tumors and whose function is to evade the immune system. Well-known examples are the receptors CTLA-4 and PD-1 that reduce the potency of TCR signaling. The fusion of their intracellular domain to a CAR also inhibits signaling, resulting in decreased cytokine production, limited lymphocyte motility and reduced target cell lysis [144].

Another hindrance to the application of CAR cell therapies is their large-scale production. The usual steps to produce CAR cells are based on extraction of cells from the patient, genetic engineering of NK or T cells, expansion and infusion in the patient. Due to the laborious process, few health care institutions are prepared to produce CAR cells. And off-site preparation of the CAR cells presents extensive logistical challenges. Thus, the production of the CAR cells is one of the principle factors that promote the high cost of this therapy. In a remarkable

research conducted by Smith and colleagues [148], they have developed an approach that may show a way around this problem. In a mouse model, they have modified the circulating T cells within the animal's own body. The strategy is based on the transfection of the CAR gene using β-amino-ester–based nanoparticles. For this, nanocarriers were coated with CD3, a lymphocyte surface antigen. The recognition of this antigen induces the endocytosis of the nanocarriers by the lymphocytes. Furthermore, peptides containing microtubule-associated sequences (MTAS) and nuclear localization signals (NLS) were added to the polymer, facilitating the rapid import of its genetic load through microtubule transport machinery. Alternative approaches include the use of viral vectors and the use of transposon/transposase systems, such as sleeping beauty, that promote integration of the CAR sequence in the host DNA [148].

Instead of a complicated scenario of transporting of patients' cells to and from specialized facilities, methodology enabling *in situ* modification of T cells implies that nanoparticles, virus and other vectors containing the CAR sequence can be produced in a central location, packaged and shipped to any hospital. All that is needed is a syringe to inject the vector into the bloodstream of the patient. As the nanoparticles are stable, this enables long-term storage, reducing the cost of this medical technology and permitting the sale of CAR cell therapies at more affordable prices.

8. Conclusions

Clearly, cancer immunotherapy can be achieved by a variety of interventions that share the common goal of boosting the antitumor immune response. These modalities may target distinct points along the cancer immunity cycle, from inducing immunogenic cell death, promoting antigen presentation and culminating in activation of innate and adaptive responses, including cytolytic T cell activity, which can then further promote antitumor immunity since tumor cell killing would reinitiate and propagate the cycle [149]. Moreover, distinct points in the cancer immunity cycle may be targeted simultaneously, enhancing even more the antitumor response.

As shown here, gene transfer plays a critical role in several key cancer immunotherapies. Vaccines, suicide gene therapy, simultaneous induction of cell death and immune response, OV and CAR-T cells all benefit from gene transfer. While the gene transfer technology will continue to evolve, the therapeutic benefit of genetically modifying cells in order to alter their function will certainly continue to be a central theme in cancer immunotherapy. The approval of Imlygic (FDA and EMA), Yescarta and Kymriah (FDA and EMA), as well as the commercialization of Oncorine (China) show that immunotherapies involving some component of gene transfer are now well established.

In addition, we expect that future approaches will rely on multiple immunotherapies that work in harmony. For example, checkpoint blockade along with the gene transfer interventions should bring about strategic combinations of inducing cell death, tumor-specific immune response and maintenance of cytolytic T cell activity. Challenges remain to be addressed, such as avoiding adverse effects, proper monitoring criteria, identification of adequate biomarkers

and definition of a reasonable price tag for cutting edge, personalized interventions. Thus, immunotherapies require further study. As such future developments unfold, gene transfer technologies are expected to remain as crucial components of cancer immunotherapy.

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Conflict of interest

The authors have no conflicts of interest.

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