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Immuno-gene therapy approaches for cancer: from *in vitro* studies to clinical trials

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1. ABSTRACT

Immunotherapy against cancer basically aims at either broadly stimulating the immune system or at engineering an immune response against a targeted tumor associated antigen (TAA). In this review, we focus on the translation of immuno-gene therapy strategies into clinical trials for various cancers. Rather than being an exhaustive compendium of the literature, the focus of this article is to underline how anti-cancer immunotherapy strategies have evolved recently. Previously, studies have used different vectors to either express immuno-stimulatory molecules or a targeted TAA. Investigators are now directing efforts to both target a TAA and to stimulate the immune system by direct or viral administration of cytokines or co-stimulatory molecules. Some groups have also tried to combine genetic immunotherapy with chemotherapy, and results have been encouraging. This novel concept might open new perspectives for the treatment of patients with advanced-stage cancer.

2. INTRODUCTION

Despite improvements in conventional therapies, cancer remains a leading cause of mortality worldwide. Immunotherapy is a promising alternative modality. The approach arose from the concept that the immune system's role is not only to protect the body from infections, but also to help to prevent the development of tumors. This second role for the immune system, also called "immunosurveillance", is the one that immunotherapy aims to enhance. The fact that many tumors have the ability to escape this sentinel function reveals the complex nature of the interactions between tumors and immune system. Thus, a clear understanding of these interactions will be needed in order to generate fully effective anti-cancer vaccines.

A T cell response is essential for anti-tumor immunity. In order to implement this response, three signals are required: (1) recognition of an antigen as a

peptide presented in the context of MHC molecules by a T cell receptor (TCR), (2) co-stimulation by appropriate accessory molecules, and (3) an inflammatory signal, called a "danger signal". Towards the goal of generating or strengthening immunity against tumors, different strategies have been employed. These approaches can be classified into two main groups: passive therapies where patients are treated with a molecule (e.g. cytokines or antibodies) and active strategies where the goal is to stimulate a patient's immune system to adequately respond to the tumor. In this review, we will focus on the second group of approaches.

Among active therapies, some are non-specific and mainly provide a co-stimulatory or an inflammatory signal using a wide range of plasmid and/or viral vectors to enhance the immune response against tumors. Other strategies aim to present tumor-associated antigens (TAAs) to the immune system in an appropriate context in order to generate a response against these antigens. In this manifestation, cell-based strategies have emerged that directly use cells of the immune system, mainly T lymphocytes or dendritic cells (DCs). DCs are potent antigen-presenting cells (APCs) that are able to prime T cells to become either effector cytotoxic T lymphocytes (CTLs) or T helper (Th) cells to induce both cellular and humoral immunity. There are at least two different ways to use DCs to activate or enhance such a response against a targeted TAA. DCs can either be "loaded" with peptides or transduced with a virus expressing a specific TAA. In the latter method, the putative TAA is fully processed internally and peptides derived from that factor are presented in the context of MHC molecules.

This review will focus exclusively on gene transfer-based approaches for immunotherapy that employ plasmid or viral vectors to engineer expression of a transgene. Although this area of research has gained considerable attention, most strategies that have demonstrated substantial efficacy in preclinical models have yielded disappointing results when translated to humans.

We report on a number of representative examples of immuno-gene therapy approaches tested recently for which proof-of-principle studies have been performed *in vitro* using human cells, and that are now making their way into clinical trials. We will then examine the outcomes of the major therapies tested in human studies. In the end, synthesis of the existing observed outcomes together may direct us towards trends that employ combined immunotherapeutic strategies.

3. DNA VACCINES

Plasmids are circular rings of double-stranded DNA that exist extra-chromosomally in bacteria. Plasmid DNA constructs can be created and manipulated easily and quickly. Moreover, very high quantities of plasmid can be produced *in vitro*. In 1993, Ulmer *et al.* showed the immunological potential of these agents by demonstrating the ability of plasmid DNA to engineer protective CD8+ CTL against a plasmid-encoded protein, in this case an influenza virus protein (1). This outcome established the theoretical foundation for the use of DNA vaccines in order to induce or heighten immune responses, and suggested their potential use for generating immunity against TAAs. Regarding cancer immunotherapy, the capability of naked DNA vaccinations to induce protective immune responses against tumor challenge in mice has been demonstrated in several studies (as reviewed for example by Restifo *et al.* in 2000 (2) and Liu *et al.* in 2003 (3)). One of the first studies used a plasmid encoding for the carcinoembryonic antigen (CEA), which is over-expressed in colorectal cancers, non-small cell lung cancers, and approximately half of breast cancers (4). Such a construct conferred immunoprotection against challenge with syngenic CEA-transduced carcinoma cells as early as 3 weeks after vaccination (4). Anti-tumor immunity following plasmid vaccination was also obtained when p53 (5) and Neu (6) were targeted. These encouraging results have led to translation of DNA vaccination into clinical trials.

Overall results from human clinical trials using plasmid DNA alone demonstrate safety but a limited ability to induce clinically meaningful anti-tumor immunity. Timmerman *et al.* have used a naked DNA vaccine to target the variable region (Id) of the B-cell lymphoma tumor-specific immunoglobulin in a Phase I/II clinical trial involving 12 patients with follicular B-cell lymphoma in remission after chemotherapy (7). Following intramuscular injections (IM) of the plasmid vaccine, no significant side effects or toxicities were observed, with half of the patients demonstrating humoral and/or T cell response to Id. One patient had an objective reduction in tumor burden during the study. Rosenberg *et al.* have completed a DNA vaccine trial targeting the gp100 melanoma-melanocyte differentiation antigen that showed more modest results (8). In that study, 22 patients with metastatic melanoma were treated; 10 received the naked DNA intradermally (ID) and 12 received it intramuscularly. Such vaccination resulted in only a partial response in one patient, and none of the patients tested exhibited evidence of the development of an anti-gp100 cellular response (8).

Despite somewhat modest outcomes, naked DNA vaccines remain a practical and promising strategy due to their safety, specificity, and relatively low cost of production (3). More recent strategies have focused on enhancing the immunogenicity of DNA vaccines. One way to enhance naked DNA vaccine potency is to use cytokines such as IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) as adjuvants in parallel. This strategy has been used in a clinical trial of a plasmid vaccine encoding the prostate-specific antigen (PSA), a well-known prostate TAA, in patients with hormone-refractory prostate cancer (9). A rise in anti-PSA IgG as well as a PSA-specific cellular immune response without systemic toxicity was observed in 2 of the 3 patients that received the highest plasmid dose. Although the use of cytokines to enhance the immunogenicity of plasmid vaccines has not been thoroughly tested yet, the results of this preliminary study are encouraging. Studies employing combination strategies have also been performed in mice models. For example, a plasmid encoding MUC1 (mucin,

Table 1. Representative examples of recent clinical trials involving the use of plasmid vaccines against cancer

	Transgene	Targeted cancer	Main results	Ref
Clinical studies	Id of the B-cell lymphoma tumor-specific Ig	Follicular B-cell lymphoma	No significant toxicity was observed. 1/2 patients showed humoral and/or T-cell response to Id.	(7)
	gp100	Metastatic melanoma	No anti-gp100 cellular response was detected.	(8)
	PSA (+IL-2, GM-CSF as adjuvants)	Hormone-refractory prostate cancer	A rise in anti-PSA IgG as well as a PSA-specific cellular immune response in 2/3 patients that received the highest dose were obtained, without toxicity.	(9)

Id: variable region; Ig: immunonoglobulin; gp100: Glycoprotein 100; PSA: Prostate Specific Antigen; IL: interleukin; GM-CSF: Granulocyte/Macrophage Colony Stimulating Factors.

which is over-expressed and exhibits aberrant glycosylation patterns in malignant cells), and IL-18 together broke tolerance to MUC1 and induced antigen-specific immunity with protective and therapeutic benefit in a murine model of pulmonary metastatic disease (10).

In summary, despite the advantages of their fundamental simplicity and absence of toxicity by themselves, the use of naked DNA vaccines to engineer an immune response against targeted TAAs have presented obstacles regarding their low immunogenicity (see Table 1). Intradermal injections of naked DNA might be more efficient than intramuscular injections to induce immune responses (11). However, optimization of the route of injection may not be able to overcome the weak immunogenicity of plasmid vectors themselves. As a matter of fact, one possible explanation for the weak immunogenicity of plasmid is that, unlike viral vectors, plasmid vaccines lack proteins as protection from degradation and to facilitate entry into host cells. Nevertheless, the emerging use of cytokines as adjuvants could help overcome this drawback with not only naked DNA vaccines but other vaccine types as well. On the other hand, a very recent study showed the potential potency of the use of plasmid vectors in a non-specific anti-cancer immunotherapy strategy (12): Mahvi *et al.* obtained tumor size regression of greater than 30% in 5 of the 12 patients treated by intra-tumoral injection of a plasmid encoding for IL-12 (12).

4. VIRUSES USED IN IMMUNOTHERAPY AGAINST CANCER

Viruses are microscopic infectious particles which need a host cell to replicate. They contain either RNA- or DNA-based genomes, and can be used as delivery vectors for gene therapy. In this context, genes encoding viral structural components are introduced separately from the viral backbone by helper plasmids and the genes required for viral self-replication are deleted or modified. These alterations not only reduce the chances of creation of a wild-type virus by recombination, they also allow for the introduction of a transgene expression cassette into the vector backbone. Transfection to packaging cell lines of the modified backbone plasmid now containing the transgene together with the helper plasmids engineers recombinant virion production. When recombinant viral vectors are used for immunotherapy against cancer, the transgene can encode either for a stimulator of the immune system or for a TAA, or, in more recent studies, both factors.

Viral vectors offer two main advantages in the context of immunotherapy. They engineer higher levels of transgene expression than levels achieved by plasmid DNA vectors, and they are often immune stimulating in themselves. Moreover, viral vectors such as adenoviruses can provide inflammatory signals, which may augment the induction of an efficient CTL immune response. We will first briefly describe the different viral vector systems commonly used in immuno-gene therapy for cancers. Note that some of these vectors are described in more detail by Mossoba *et al* (13) in the context of the examination of outcomes mediated by dendritic cell-based cancer immunotherapy. The results of representative studies performed with the cited viral vectors will be reported in the next sections of this review, classified based on the delivery strategy used.

4.1. Poxviruses

Poxviruses are linear double-stranded DNA viruses that have been used in immuno-gene therapy strategies, in part, because safety of their use has been extensively demonstrated in humans. The modified vaccinia Ankara (MVA) virus was isolated after over 500 passages that led to a loss of approximately 31 kb of its genome (14). As a consequence, the viral replication functions are impaired, which is a key factor for safety of administration. Recombinant vaccinia can carry over 25 kb of foreign DNA. The recombinant Canarypox virus ALVAC and Fowlpox (rF) attenuated viruses were derived in a similar manner and have been found to be safe as well. Indeed, between 1989 and 2004, 82 poxvirus-based gene therapy clinical trials were carried out (15).

4.2. Adenoviruses (Ad)

Adenoviruses are linear non-enveloped double-stranded DNA viruses that have cloning capacity of up to 35 kb. They have a very efficient nuclear entry mechanism and engineer high level transient transgene expression. In theory, their strong inherent immunogenicity could act as an adjuvant but may also be a drawback with respect to tissue inflammation as well as premature clearance of the vector by the host. Pre-existing serotype-specific neutralizing antibodies against Ad particles can prevent successful re-application of the vector. Approaches to overcome this hurdle include the sequential use of vectors based on different serotypes (e.g., Ad2 and Ad5), and the use of vectors based on animal adenoviruses (e.g., canine or ovine Ad) (16). Different regions of the genome were deleted in first-generation Ad-vectors to prevent viral replication, and also in second- and third- generation Ad vectors to reduce their immunogenicity (16). There were 240 Ad-vectors gene therapy trials reported between 1989 and 2004 (15).

4.3. Adeno-associated viruses (AAVs)

AAVs are single-stranded DNA viruses that also engineer high levels of transgene expression. AAVs are somewhat difficult to produce as they have an obligate requirement for a helper virus. They can only carry up to 5 kb of foreign DNA. Also, transduction efficiencies using these viruses tend to be highly variable. The use of AAV vectors is motivated by the persistence of this non-pathogenic virus in the host cell and sustainable therapeutic gene expression (17). They can also be produced at very high titers. AAV vectors have been used in 19 gene therapy clinical trials between 1989 and 2004 (15).

4.4. Retroviruses

Retroviruses are linear single-stranded RNA viruses able to generate long-term expression of the transgene. After they enter the host cell, their genome is reverse-transcribed into double-stranded DNA and integrated into the host genome. Two types of retroviruses have mainly been used in gene therapy: oncoretroviruses and lentiviruses.

Oncoretroviruses are well-known and one of the first viruses used in gene therapy applications. However, some recent outcomes call into question the safety of using these vectors. In one study, they have been linked to oncogenesis (18) in a clinical trial involving X-linked SCID patients (19), where the vector insertion may have contributed to the subsequent development of leukemia in some patients. It should be noted, however, that as yet an analogous clinical trial in Britain has not reported a similar outcome (20, 21).

Lentiviruses (LVs) also belong to the retroviridae family. They are also very efficient gene transfer agents. LVs can be pseudotyped to infect a number of cell types, and unlike oncoretroviruses, they are able to infect slowly dividing cells. Moreover, advances in LV design, detailed safety analyses, and long-term testing in gene therapy approaches will increase their likelihood for wide-spread clinical utilization (22). On the other hand, considering the broad academic interest in their use, only a few studies as yet have used LV for cancer immuno-gene therapy; for example, the *ex vivo* transduction of DCs with melanoma TAAs (23, 24), antigen presentation for CTL responses (23), and transduction of CD34+ cell-derived DCs for HIV/AIDS immunotherapy (25,26). This vector system also allows the ability to engineer co-expression of more than one gene. For example, other effector genes like cytokines or co-stimulatory molecules can be co-expressed along with a TAA, which could potentially improve the potency of the immune response (27).

In summary, there is a wide panel of viral vectors with different characteristics regarding their safety, immunogenicity, ability to infect cells, and capacity to express transgenes. This variety gives investigators many options for their use in the context of immunotherapy against cancer by direct injection or to transduce cells (tumor cells or cells of the immune system).

5. DIRECT INJECTION OF VIRUSES AS VACCINES

As mentioned above, one method for implementation of vectors for anti-cancer vaccines is by direct intravenous (IV) or IM administration to patients of recombinant viruses that are modified to engineer expression of TAAs, co-stimulatory molecules, pro-inflammatory cytokines, or combinations of these factors. Indeed, encouraging results have been obtained using viruses to express cytokines towards the goal of activating non-specific immune responses that can be effective against tumors. In one clinical trial, administration of a recombinant adenovirus that engineered IL-12 expression in solid tumors of patients with metastatic melanoma led to several disease stabilizations with the highest vector dose (28). Similarly, a recombinant adenovirus that engineered expression of IFN-□amma was used in a clinical study involving patients with advanced primary cutaneous T cell lymphomas or multi-lesional cutaneous B cell lymphomas. Of the 10 patients evaluated in that study, 4 showed complete responses and 2 showed partial responses (28).

Most viral vaccine-based strategies, however, aim at inducing or augmenting a specific immune response against TAAs. For example, the tumor suppressor protein p53 is a frequently targeted TAA. Mutations in the p53 gene are a common genetic alteration in tumors and lead to the production of a defective protein with a significantly longer half-life than the normal protein (29). This results in uncontrolled cellular proliferation that can cause tumor formation. In one clinical study published in 2003, the outcome of intravenous vaccination with a canarypox virus encoding wild-type p53 in mutated p53-overexpressing colorectal cancer patients was assessed (30). Such a vaccination schema led to a measurable immune response against p53 without serious systemic toxicity but only one patient showed a clinical response. In an assessment of this outcome, the authors suggest the need for a secondary vaccine to potentially enhance clinical efficacy (30).

More recently, the trend has been for investigators to co-express cytokines or co-stimulatory molecules along with the targeted TAA to try to enhance the anti-tumor immune response using viral vaccines. In pre-clinical models, this strategy seems to indeed demonstrate a more pronounced efficacy than when using TAAs alone. In a clinical trial targeting the TAA MUC1, Rochlitz *et al.* tested a highly attenuated vaccinia vector (MVA) encoding IL-2 and MUC1 (31) in patients with different solid tumors. Repeated intramuscular injection with increasing doses of the viral suspension was well tolerated and resulted in transient disease stabilization in several patients. The same group used a similar strategy in a clinical study involving MUC1-positive patients with advanced prostate cancer (32). One of the 16 patients in that trial demonstrated an objective tumor response. Importantly, none of the patients in that study showed further tumor progression throughout the study duration. The PSA level of two patients was also stabilized for more than one year. Another vector based on the same schema is being tested with IL-2 and human papilloma virus E6 and E7 proteins as the targeted TAA instead of MUC1 (28).

Table 2. Representative examples of recent clinical trials involving direct viral injections as vaccines against cancer

	Virus and Transgene	Route	Targeted cancer	Main results	Reference
Clinical studies	Ad-IL12	IT	Metastatic melanoma	Several disease stabilizations with the highest dose.	(28)
	Ad-IFN-□amma	IT	Cutaneous T and B cell lymphomas	Of 10 patients, 4 showed complete responses and 2 showed partial responses.	(28)
	ALVAC-p53	IV	Colorectal cancer	An immune response against p53 without serious toxicity was shown but only one patient had stable disease.	(30)
	MVA-IL12,MUC1	IM	MUC1+	The treatment was well tolerated, and transient disease stabilization was obtained in several patients.	(31)
	MVA-IL12,MUC1	IM	Prostate cancer	One of the 16 patients had an objective tumor response. None of the patients showed tumor progression and the PSA level of two patients was stabilized for more than one year.	(32)
	ALVAC-CEA,B7.1	IM	CEA+ adenocarcinoma	The safety of the approach was demonstrated; 20% of the participants had stabilization of the disease after four injections. The stabilization was associated with the induction of a CEA-specific T-cell response.	(34)

Ad: Adenovirus; ALVAC: Canary-pox virus; MVA: Modified Vaccine Ankara; IL: interleukin; IFN: interferon; MUC: mucin; CEA: CarcinoEmbryonic Antigen; PSA: Prostate Specific Antigen; IT: intratumoral; IV: intraveinous; IM: intramuscular

Other studies have used co-expression of a co-stimulatory molecule to provide for the "second signal" in the immune activation cascade. This is the inflammatory signal that, in addition to antigen recognition, is required for the generation of efficient cytotoxic T effector cells. Interesting data were previously obtained using intramuscular delivery of a recombinant canarypoxvirus (ALVAC) that engineered expression of CEA as a TAA along with the costimulatory molecule B7.1 in a pilot study (33) and also in a Phase I clinical trial (34). In both cases, the relative safety of this combined approach was demonstrated. Indeed, 20% of the participants in total had stabilization of the disease after four injections of the recombinant vector. In addition, the disease stabilization was associated with the induction of a CEA-specific T cell response (34).

Taken together (see Table 2), at a minimum the studies mentioned above present evidence of the safety of administration of different viral vectors to humans. More promising is the fact that several studies using viruses co-expressing a TAA along with a stimulatory molecule have been shown to lead to cancer disease stabilizations. Nevertheless, at this stage of progression of this therapeutic modality, tumor objective responses are rare, underlying the requirement for improvement of this method or its combination with other strategies.

6. AUTOLOGOUS TUMOR VACCINES

Another application of viral vectors in cancer immunotherapy is to transduce target cells *ex vivo*. Irradiated autologous tumor cells transduced to express immuno-stimulatory molecules represent an original class of immunotherapy-based anti-cancer vaccines. As early as the 1990's, several *in vivo* studies in murine models have shown that cancer cells modified to secrete cytokines by *ex vivo* gene transfer are able to generate anti-tumor immunity. This approach has been extensively tested by Dranoff *et al,* who examined the outcome of vaccination by tumors transduced with retroviruses encoding a panel of potential immunomodulators in a B16 melanoma model (35). The most potent, long-lasting, and specific anti-tumor immunity was obtained with cells expressing GM-CSF (35). This strategy has also shown inhibition of tumor growth (36) and elimination of pre-existing tumors in some other models (37).

In human studies, a human glioma-derived cell line was retrovirally transduced to express three molecules chosen for their ability to induce and enhance immunity. These molecules included B7-2 (a co-stimulatory molecule mainly present on mature DCs), GM-CSF (an activator of APCs), and IL-12 (a proinflammatory cytokine involved in cross-talk between innate and adaptative immune arms). Transduced cells were able to induce an increased anti-tumor cytotoxicity *in vitro* when cultured with T cells (38). Unfortunately, when the same group tested a similar strategy using autologous tumor cells expressing B7.2 and GM-CSF as a vaccine in a pilot clinical trial, they encountered significant technical hurdles (39). From 116 malignant glioma and 32 melanoma patients, they were only able to prepare vaccines for 5 glioma and 3 melanoma patients. In addition, no specific anti-tumor immunity was demonstrated although an inflammatory response was observed in the patients receiving the vaccines. Moreover, although only minor toxicities occurred and 3 patients had prolonged recurrence-free intervals after vaccination, disease progression was noted in 6 patients.

Clinical studies using autologous transduced tumor cells as anti-cancer vaccines have been more successful with the use of GVAX. GVAX vaccines are composed of whole tumor cells genetically modified by an adenovirus to secrete GM-CSF. This vaccine was well-tolerated and anti-tumor immunity has been shown using GVAX in a range of cancers including melanoma, prostate, pancreatic, and lung cancers (40). In one recent study, GVAX was tested in a Phase I clinical study involving Stage IV renal cancer patients. Both cellular and humoral anti-tumor immune responses were demonstrated, which may have contributed to the relatively long overall survival (41). The main limitation of this approach, however, is the necessity for genetic transduction of individual tumor cells. To circumvent this, Nemunaitis *et al.* developed in 2006 a "bystander" GVAX platform composed of autologous tumor cells mixed with an allogeneic GM-CSF-secreting cell line. It was found that GM-CSF secretion by the

Table 3. Representative examples of recent (pre)clinical trials involving autologous tumor vaccines against cancer

	Virus and Transgene	Targeted cancer	Main results	Reference
Pre-clinical studies	ORV- B7-2, GM-CSF, IL-12	Glioma	An increased anti-tumor cytotoxicity was measured.	(38)
Clinical studies	ORV- B7-2, GM-CSF, IL-12	Malignant glioma and melanoma	Technical hurdles to prepare the vaccines were encountered. No specific anti-tumor immunity was demonstrated. 3 patients had prolonged recurrence-free intervals after vaccination, all 6 patients died.	(39)
	GVAX: Ad-GM-CSF	Renal tumors	Anti-tumor cellular and humoral immune responses were shown that may have contributed to lengthened survival.	(41)
	"bystander" GVAX (mixed with an allogeneic GM-CSF-secreting cell line)	Non-small-cell lung	No objective tumor response was observed. The survival was less favorable than in GVAX clinical trials.	(42)

ORV: Onco-retrovirus; GVAX: whole tumor cells genetically modified by an adenovirus (Ad) to secrete GM-CSF; IL: interleukin

vaccine was higher with this method (42). However, when tested in a Phase I/II clinical trial involving patients with advanced-stage non-small-cell lung cancer (NSLC), no objective tumor responses were seen (43). Survival was also less favorable compared to what was obtained with the previous clinical study using GVAX for treating patients with NSLC (43).

Mechanisms involved in this approach have been further studied by E Jaffee's group. In a Phase I clinical trial using GM-CSF-secreting tumor cells as a vaccine in patients with metastatic renal cell carcinoma, they analyzed the $CD8^+$ T cell response in the patients that showed the greatest magnitude of delayed-type hypersensitivity as well as strong clinical responses (44). The results suggested that paracrine GM-CSF tumor vaccines might generate a diverse repertoire of $CD8^+$ T cell responses (44).

Overall, despite encouraging preliminary results (see Table 3), autologous tumor vaccines have not yet been used successfully in diverse settings. Improvements in technical aspects related to autologous tumor cell transductions may overcome some of these limitations.

7. T LYMPHOCYTES-BASED VACCINES

Another way to use viruses in the context of immunotherapy is to directly transduce cells of the immune system. As mentioned above, one strategy is to transduce T lymphocytes. Indeed, T lymphocytes are responsible for the cellular immune response and the memory response that both have key roles in cancer therapy. Recent studies have used oncoretroviruses to modify T lymphocytes to engineer expression of a TCR against a pre-determined TAA. This confers a novel anti-tumor specificity due to the induced capacity of the T cells for recognizing the targeted TAA. Some of these studies will be discussed below.

In 2003, Morgan *et al.* showed *in vitro* that genetic modification of T lymphocytes by a recombinant oncoretrovirus induced expression of the anti-glycoprotein 100 (gp100) TCR, which afforded avid recognition of melanoma tumor antigen gp100 (45). In addition, transduced tumor-infiltrating lymphocytes maintained their pre-existing reactivity against non-gp100 autologous melanoma antigens. The concept of T lymphocyte-based immunotherapy as a platform is also supported by data published by Zhao *et al.* in 2005, showing that oncoretrovirally-transduced T lymphocytes expressing the TCR specific for the cancer-testis antigen NY-ESO-1 were able to recognize and kill various NY-ESO-1-positive tumor cell lines *in vitro* (46). A similar *in vitro* study confirmed the proof-of-principle for this approach, using T lymphocytes transduced with an oncoretroviral vector encoding a murine anti-p53 TCR (47). In that study, *in vitro* killing of a broad spectrum of human tumor cell lines was demonstrated (47).

A very interesting evolution of this T lymphocyte vaccine approach is the isolation of alpha-and □etachains of highly reactive anti-TAA TCR from T lymphocytes that mediate regression of tumors in patients. In 2005, Hughes *et al.* isolated genes for the alpha- and beta-chains of the TCR from a patient that comprise an effective anti-MART1 TCR and cloned them into oncoretroviral vectors (48). Transfer of this TCR complex by transduction equipped the T lymphocytes to be reactive against tumor cells. Finally, this immunotherapy strategy was recently tested in a clinical trial involving 15 patients with metastatic melanoma (49). Successful durable engraftment of transduced T cells was demonstrated and an objective regression of metastatic melanoma lesions was observed in 2 patients, suggesting benefit in patients with established tumors (49).

In summary, it has been shown that autologous T lymphocytes retrovirally transduced to express an anti-TAA TCR can mediate *in vitro* killing of tumor cells expressing the TAA. Further, when reinfused into cancer patients, these transduced cells can express the transgene long-term and can mediate the durable regression of established tumors (see Table 4). An advantage of this method is that T cells can be expanded to large numbers (50). However, as yet the objective response rate remains disappointingly low. This may be due to an insufficient level of transduction. Optimization of T cell transduction methods will be required before this approach can reach its full potential. The use of more powerful promoters specific to T cells could also enhance the efficacy of this strategy. Co-insertion of cytokines or tissue-homing molecules could engineer stronger immunity. The idea evocated by Zhao *et al.* of engineering a population of T cells with both Class I- and Class II-restricted TCRs may also be beneficial (46). A limitation of this strategy is the possible occurrence of chain mispairing, however, it is likely that this outcome can be prevented by modification of the TCR constant region or insertion of single-chain receptors (51).

Table 4. Representative examples of recent (pre)clinical trials involving TL-based vaccines against cancer

	Virus and Transgene	Targeted cancer	Results	Reference
Pre-clinical studies	ORV-anti-gp100TCR	Melanoma	An anti-melanoma activity was developed.	(45)
	ORV-antiNY-ESO-1TCR	Various cancers	Recognition and killing of various NY-ESO-1-positive tumor cell lines was obtained.	(46)
	ORV- anti-p53 TCR	Various cancers	*In vitro* killing of a broad spectrum of human tumor cell lines was demonstrated.	(47)
Clinical studies	ORV-anti-MART-1 TCR	Metastatic melanoma	Successful durable engraftment was demonstrated and objective regression of metastatic melanoma lesions was observed in 2/15 patients.	(49)

ORV: oncoretrovirus; TCR: T-Cell Receptor; gp100: Glycoprotein 100; MART-1: Melanoma antigen recognized by T-cells

8. DC-BASED VACCINES

Another promising genetic cancer immunotherapy strategy uses the transduction of autologous dendritic cells (DCs) by a virus expressing a targeted TAA (13, 52). DCs are the most potent antigen-presenting cells in the immune system. They are able to initiate and sustain strong immune responses through presentation of processed antigens in the form of peptides bound to MHC molecules. After migrating to secondary lymphoid organs, DCs prime resident T cells to become activated effector cytotoxic T lymphocytes (CTL) or T helper cells for inducing both cellular and humoral immunity. In addition, they allow the formation of memory T and B cells for later recall responses. Since the year 2000, many studies have been published using different viruses for DC transduction. A sampling of these is highlighted below:

8.1. Poxvirus DC vaccines

Several poxviruses have been used for DC-based cancer immunotherapy. Recombinant canarypox viruses (ALVAC) have the characteristic of inducing apoptosis in infected cells. Apoptotic, virally-infected DCs can be infused into patients and be taken up by uninfected DCs, resulting in efficient cross-presentation of the virally introduced antigen. Motta *et al* observed this phenomena in a study, in which they evaluated the use of DCs transduced with ALVAC-MART-1 against melanoma *in vitro* (53). There, a MART-1-specific T cell immune response was demonstrated, indicating the efficiency of this method for engineering antigen presentation. Despite this interesting and promising outcome, to our knowledge this virus has not yet been used in a clinical trial as a DC-based anti-cancer vaccine.

The modified vaccinia Ankara virus (MVA) has a proven immunologic efficacy when used as a gene transfer tool (54). Safety of vaccination with modified MVA-transduced DCs has also been recently demonstrated in a Phase I clinical trial involving Stage IV melanoma patients (55). In that study, a T cell response against the targeted TAA, tyrosinase, was shown. However, only a partial tumor response in one patient was observed. Although this study showed safety and bioactivity for this vaccine, clinical benefits need to be improved, perhaps by optimizing the doses and timing of the vector-transduced cells.

In the context of immunotherapy, AAV has the advantage of engineering fairly high transgene expression. In 2005, Liu *et al.* (56) showed the rapid *in vitro* induction of strong BA46-specific MHC Class I-restricted CTL responses after transduction of DCs with a rAAV that engineered expression of the breast cancer-associated antigen BA46, also called lactadherin. Similarly, Mahadevan *et al.* demonstrated the generation of a T lymphocyte response against PSA after DC transduction by a recombinant AAV (57). However, these *in vitro* studies have not yet led to clinical trials to our knowledge. One potential limitation may be the fact that AAVs are, by nature, difficult to produce in a clinically-acceptable form.

Fowlpox viruses cannot replicate in infected mammalian cells but can engineer expression of their transgene for 14 to 21 days. Morse *et al.* showed in 2005 (58) the safety and potency of using DCs transduced with a fowlpox virus co-expressing CEA, a TAA overexpressed in a number of cancers, along with a triad of co-stimulatory molecules (B7.1, intercellular adhesion molecule-1, and leukocyte function-associated antigen-3), called TRICOM. In this clinical trial involving 14 CEA-positive cancer patients, an increase of CEA-specific T cells was measured in 10 patients. Moreover, the strongest immune responses were correlated with minor clinical benefits or with stabilization of the disease. This study showed a correlation between immune response and clinical benefits, which encouraged attempts to create cancer vaccines with higher immuno-stimulatory activities.

8.2. Oncoretrovirus DC vaccines

Oncoretroviruses can efficiently transduce DCs. For example, in an *in vivo* study describing a DC-based immunotherapy strategy targeting PSA/PSMA, we (Medin) have been able to achieve 80% functional transduction efficiencies with recombinant oncoretroviral vectors (59). In that *in vivo* murine study, we have demonstrated that antibody and cellular responses are generated following PSA and PSMA gene transfer into DCs. This response also correlated with protective immunity against specifically-engineered TRAMP-C1 prostate cancer cell tumor challenge. Along these lines, it has previously been shown that oncoretrovirally-transduced human DCs that express human epidermal growth factor receptor 2 (HER2/neu/c-erbB2), which is present at high levels in a variety of human cancers, elicit HER2-specific CTL and Th1 cells *in vitro* (60). These initial studies indicate that a recombinant oncoretrovirally-transduced DC vaccination approach may represent a future delivery modality in the immune therapy of cancer.

8.3. Lentivirus (LVs) DC vaccines

As mentioned above, LVs are promising gene therapy vectors, especially due to recent research efforts

focused on improving their safety (22). The potency of LV-transduced DCs as anti-cancer vaccines has been demonstrated in numerous *in vitro* settings. For example, in 2003, Breckpot *et al.* were able to efficiently transduce human and murine DCs with LVs engineering expression of the MAGE-A3 and OVA antigens (61). They also showed that transduced DCs could elicit antigen-specific immune responses. In another study, Lopes *et al.* generated DCs transduced with a LV expressing the melanoma antigen Melan-A (MART-1) (62). Co-culture of these transduced DCs with autologous naïve T cells led to the expansion of cells that recognized a Melan-A epitope and were functional as demonstrated by IFN-□amma release upon antigen stimulation. Although this review focuses on human studies, it should be noted that this strategy also demonstrated efficacy in different murine models (63-65). However, to our knowledge, no clinical trials of LV-DC based immunotherapy against cancer have yet been performed.

Our laboratory (Medin) has experience using LVs for genetic therapy of different diseases (66-69). We are currently developing several projects towards the goal of implementation of this vector system for immunotherapy. In one study, we have subcloned into our LV backbone the cDNA sequence for a non-signaling form of murine erbB2, corresponding to the human Her2/neu antigen that is up-regulated in many cancers. Vaccinations of animals with murine transduced DCs led to the *in vivo* production of antibodies against erbB2 and protection against specific tumor challenges (data submitted for publication). In another study, we cloned the cDNA for the CEA into a LV backbone, in collaboration with a company that can produce clinical grade vectors. The potency of transduced DCs as curative vaccines against colorectal cancer will be tested *in vivo* in CEA transgenic mice and *in vitro* using human cells, with the goal to eventually translate this approach into clinical trials.

One of the reasons that may explain the few number of clinical studies using this promising strategy is the fact that integrating vectors such as LVs can have deleterious insertional events that activate oncogenes or decrease expression of tumor suppressor genes. Improved safety mechanisms being incorporated into LVs, such as our work with a novel enzyme/prodrug suicide gene therapy combination (67), will likely increase the number of trials involving these vectors.

8.4. Adenovirus (Ad) DC vaccines

Adenoviruses are efficient gene-transfer vectors that have been tested in *in vitro* proof-of-principle studies using transduced DCs in anti-cancer models. Antigen-specific CTL responses that are able to recognize and kill tumor cells have been obtained using Ad-transduced DCs encoding for several TAAs: the human telomerase reverse transcriptase (hTERT, important in maintaining cell immortality and expressed in almost 90% of human tumors) (70); CEA which, as mentioned above is over-expressed in a number of cancers (71); and a dominant-negative form of survivine (72), which is an anti-apoptotic protein expressed at high level in almost all human cancers. Interestingly, in 2004 Schumacher *et al.* demonstrated that for the antigens MART-1 and AFP (alpha-fetoprotein, a marker of testicular cancer) Ad transduction of DCs actually made them become more mature (73). These transduced DCs consequently expand antigen-specific T cell activation to a higher degree than non-transduced peptide-pulsed DCs. In these last two studies, TAA-specific T cell responses were shown using cells from both healthy donors and cancer patients. One recognized limitation in genetic immunization strategies is that the majority of putative TAAs are essentially self human-antigens. In an effort to overcome self-tolerance to these protein targets, we have initiated clinical trials that employ a xenoantigenic vaccination strategy. In these studies, we (Foley) have prepared a clinical-grade Ad vector incorporating the cDNA for a kinase-dead rat HER-2 gene (AdrHER-2). The ability to generate anti-HER-2 responses is currently being assessed in two clinical trials where metastatic HER-2+ breast cancer patients receive either AdrHER-2 alone or CD34+-derived DCs transduced with AdrHER-2 *ex vivo*. To date both approaches have proven to be safe. Prelimary immune outcome analysis supports the benefit of using CD34+ DCs as a cellular adjuvant (unpublished data).

In conclusion, this sampling of results demonstrate that DC-transduced anti-cancer vaccines have shown their promising potency *in vitro* and are now making their way into first and second generation clinical trials (see Table 5).

9. COMBINED STRATEGIES

To date, outcomes of clinical immunotherapy strategies against cancers have had fairly disappointing results compared to the potent anti-tumor effects observed in murine models. One major reason for this is the fact that most patients involved in such studies are in very advanced stages of cancer. This places an incredible burden on immunotherapy schemas. At present investigators are trying to overcome the limited efficacy of individual approaches on late-stage diseases by combining different immuno-modulatory strategies to overcome large tumor masses or advanced metastatic disease. One idea is to combine several different immunotherapy methods either to stimulate the immune system by alternative mechanisms, or to use prime-and-boost vaccination courses to enhance the immune response. Others are attempting to combine immunotherapy with chemotherapy. In a review about colorectal carcinoma treatments, Correale *et al.* suggested that one of the main limitations of current genetic immunotherapy approaches is the appearance of effector-resistant tumor cells (74). Tandem treatment by chemotherapy could make such tumor cells more susceptible to the cytotoxic response induced by immunotherapy. Some combinations, using either multiple immunotherapy methods or immunotherapy with chemotherapy, have indeed shown improved efficacy in clinical trials, as outlined below.

Table 5. Representative examples of recent (pre)clinical trials involving DC-based vaccines against cancer

	Virus and Transgene	Targeted cancer	Results	Reference
Pre-clinical studies	ALVAC-MART-1	Melanoma	A MART-1-specific T-cell immune response was demonstrated.	(53)
	rAAV-BA46	Breast cancer	An *in vitro* induction of a strong, rapid BA46-specific MHC Class I-restricted CTLs was demonstrated.	(56)
	rAAV-PSA	Prostate cancer	A T lymphocytes response against the PSA was generated.	(57)
	ORV-HER2	Various	An HER2-specific CTL and Th1 cells were elicited *in vitro*.	(60)
	LV-MAGE-A3	Various	Elicited antigen-specific immune responses *in vitro*.	(61)
	LV-MART1	Melanoma	Functional T-cells that recognize the Melan-A epitope exanded.	(62)
	Ad-hTERT Ad-CEA Ad-survivin	Various	For all 3, TAA-specific CTL able to recognize and kill tumor cells was engineered.	(70) (71) (72)
	Ad-MART1 Ad-AFP	Various	Same *in vitro* results but from both healthy donors and cancer patients cells were obtained.	(73)
Clinical studies	MVA-hTyr	Melanoma	A T cell response against the tyrosinase, was shown but only one partial response in only one of 6 patients was observed.	(55)
	rF-CEA-TRICOM	Various CEA+	An increase of the CEA-specific T cells number was measured in 10/14 patients. Highest peaks were correlated with a minor response or a stabilization of the disease. the disease	(58)
	Ad-p53	Small cell lung cancer	A p53 specific CTL response was observed in 57.1% of the 29 patients. One patient showed a partial clinical response and 7 patients had stable disease. When vaccination was followed by second-line chemotherapy, 61.9% of patients showed an objective clinical response, whereas the usual response rate in a similar population is 6% to 16%.	(76)

ALVAC: Canary-pox virus; rAAV: Adeno-Associated Virus; ORV: Onco-RetroVirus; LV: LentiVirus; Ad: Adenovirus; MVA: Modified Vaccine Ankara; rF: Fowlpox virus; MART-1: Melanoma antigen recognized by T-cells; BA46: Breast cancer associated antigen; PSA: Prostate Specific Antigen; HER2: Human Epidermal growth factor Receptor 2; MAGE: Melanoma antigen recognized by T-cells; hTERT: Human Telomerase Reverse Transcriptase; CEA: CarcinoEmbryonic Antigen; AFP: alpha fetoprotein, marker of testicular cancer; hTyr: human tyrosinase; TRICOM: triade of co-stimulatory molecules

Regarding approaches combining genetic immunotherapy with chemotherapy, a relevant example is a DC-based vaccine with p53 as the chosen TAA, that was shown to be successful *in vitro* by Nikitina *et al.* in 2001 (75). This vaccine was then tested in combination with chemotherapy in a clinical trial involving 29 patients with extensive stage small cell lung cancer (76). Although a p53-specific CTL response was observed in 57% of patients, only one patient showed a partial clinical response and 7 patients were found to develop stable disease. However, when vaccination was followed by second-line cytotoxic chemotherapy involving carboplatin/VP-16, cisplatin/VP-16, or cisplatin/CPT-11, 62% of patients showed an objective clinical response, whereas the usual responses rate in a similar population is 6-16%. This study suggests that the combination of cancer immunotherapy and chemotherapy might provide significant benefit.

Combined administrations of viral vectors have been tested for prime-and-boost effects as well as in combination with chemotherapy. In 2005, Marshall *et al.* published results of a clinical trial targeting CEA involving 58 patients with advanced CEA-expressing cancers (77). These investigators tested the combined effects of recombinant viruses expressing CEA and co-stimulatory molecules: fowlpox (rF)-CEA(6D)-TRICOM and vaccinia (rV)-CEA(6D)-TRICOM, along with GM-CSF. Towards this goal, they designed their patient population to contain 8 cohorts. In 3 cohorts, they used 3 different doses of fowlpox virus, and in 3 other cohorts they used 3 different doses of vaccinia virus followed by fowlpox booster vaccinations. In the last 2 cohorts, they used the same prime-and-boost strategy but added different doses of GM-CSF to the treatment. Results demonstrated the safety of all of these combinations and the development of a specific immune response against CEA (72). Moreover, the disorder stabilized for more than 4 months in 23 patients. A trend for an increased overall survival in patients receiving both rV and rF compared to patients receiving rF only was observed. There was also a trend towards longer progression-free survival in patients additionally receiving GM-CSF. These encouraging outcomes must be confirmed in larger studies.

Another combination has shown relevant immunological effects against prostate cancer; here targeting PSA. A rV-PSA vaccine induced specific immune responses without toxicity in a Phase I clinical trial (78). However, in that trial, no clinical modulations were observed. In 2006, Arlen *et al.* tried combining the chemotherapy drug docetaxel with immunotherapy by vaccinating docetaxel-treated patients first with rV-PSA mixed with rV-B7.1 (coding for the named costimulatory molecule), followed by booster vaccination with rF-PSA, each time with tandem injections of GM-CSF (79). This combination of immunotherapy with chemotherapy did not cause any toxicity. Moreover, the vaccine may have improved response duration while on docetaxel, based on a comparison with a historical control of patients receiving docetaxel alone. Larger studies are also required to confirm these promising observations.

It should be noted that the use of chemotherapy along with immunotherapy is not limited to only genetic approaches for immunotherapy. Such combinations have also been shown to allow longer post-chemotherapy recurrence times and greater survival for patients with glioma vaccinated with loaded DCs (80). The idea of combining strategies to fight cancer is relatively recent. However, the few studies relating trials of combinatory treatments showed promising trends to improved efficacy that have, in some cases, to be validated in larger controlled trials.

10. CONCLUSION

Immunotherapy against cancer has been built on a foundation of two strategies. 1) Non-specific therapies aimed at broadly stimulating the immune system. 2) Specific strategies aimed at engineering an immune response against a targeted TAA. Many studies use different plasmid or viral vectors to express either immuno-stimulatory molecules or a targeted TAA. In particular, vaccinations with virally-transduced autologous cells (tumor cells or cells of the immune system) have shown to be efficient at inducing anti-tumor immunity *in vitro* and in *in vivo* murine models.

However, a main obstacle encountered for the application of these strategies that have given encouraging pre-clinical results is the fact that few clinical trials are arising. There is a need for more interactions between basic scientists and front-line physicians to allow for an easier transition to clinical trials.

In this review, we focused on representative examples that involve translation of immuno-gene therapy strategies to clinical trials for various cancers. A recent trend in the field is a move towards more complex approaches. Investigators are now directing efforts to both target a TAA and to further stimulate the immune system by direct or viral administration of cytokines or co-stimulatory molecules. There is also a trend to use priming vaccination to break the tolerance or better present danger signals, before boosting vaccinations. Finally, some groups have tried to combine genetic immunotherapy and chemotherapy, with encouraging results. Although it has to be better established, this novel concept might open new perspectives for the treatment of patients with advanced-stage cancer. Researchers are also testing ways to interfere with immuno-evasion mechanisms (81). The new generation of anti-cancer vaccines may involve both stimulation of tumor-specific immunity and inhibition of tumor-induced tolerance (81).

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Abbreviations: Ad: Adenovirus; AAV: adeno-associated virus; AFP: alpha-fetoprotein; ALVAC: Canary-pox virus; APC: antigen presenting cell; BA46: Breast cancer associated antigen; CEA: Carcinoembryonic antigen; CTL: cytotoxic T lymphocytes; DC: dendritic cell; GM-CSF: granulocyte/macrophage colony stimulating factors; gp100: glycoprotein 100; HER2: Human epidermal growth factor receptor 2; hTERT: human telomere reverse transcriptase; hTyr: human tyrosinase; ID: intradermal; IFN: interferon; Ig: immunoglobulin; IL: interleukin; IM: intramuscular; IT: intratumoral; IV: intraveinous; LV: lentivirus; MAGE: Melanoma antigen; MART-1: Melanoma antigen; MUC: mucin; MVA: Modified vaccine ankara; ORV: onco-retrovirus; PSA: Prostate specific antigen; rF: Fowlpox virus; TRICOM: triade of co-stimulatory molecules; TAA: Tumor associated antigen

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