

Antitumor Gene Immunotherapy in Melanoma

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ABSTRACT

Melanoma is probably the most immunogenic cancer. The immunotherapy interest has increased since three new immunotherapy drugs for malignant melanoma have been approved by the FDA: an anti-CTLA4 antibody called ipilimumab (Yervoy®) and anti-PD1 antibodies called pembrolizumab (Keytruda®) and nivolumab (Opdivo®). In spite of the great advance of these drugs for patients with advanced melanoma, only around 10-15% of these patients respond to these treatments. In this context, gene therapy can be used to improve the immune antitumor response. In the field of immunotherapy, the different strategies of gene therapy are: gene vaccines (DNA or RNA vaccines), genetically modified cell vaccines (tumor cells or dendritic cells), virotherapy (genetically modified oncolytic virus), gene silencing of immune checkpoint molecules and genetically engineered T cells and chimeric antigen receptors (CAR)-T cell therapy. In this chapter, the use of the different strategies of genetic immunotherapy for malignant melanoma mentioned are reviewed. This review collects some of the most important assays of each of these genetic immunotherapy strategies.

Keywords: Melanoma; Immunotherapy; Gene therapy; Tumor vaccines; Oncolytic virus; CAR

INTRODUCTION

The immune system can recognize tumor associated antigens (TAA) and trigger an antitumor immune response. This phenomenon is known as immunosurveillance. However, tumor cells have a series of mechanisms that allow tumor avoidance of immune response and finally the cancer progresses. The failure of the antitumor response is due at least in part to the immunosuppressive tumor environment and the consequent suboptimal antigen presentation. The main cells implicated in this immunosuppressive response are T regulatory cells (Treg). Treg express the co-inhibitory molecule CTLA4 and the nuclear transcription factor Foxp3. CTLA4 binds B7.1/ B7.2 from the antigen presenting cell (APC) surface and blocks the necessary co-stimulatory signal for an optimal antigen presentation. Foxp3 is critical for Treg development and function [1]. These cells secrete the immunosuppressive cytokines IL-10 and TGF- β . Furthermore, after antigen recognition the T cell also expresses CTLA4 on its surface. On the other hand, tumor cells express PD1-L on their surface which binds PD1 from T cell membrane and inactivates this cell contributing to the tumor escape.

Melanoma is probably the most immunogenic cancer since it has been reported spontaneous tumor regression [2], relation between better prognosis and presence of tumor infiltrated lymphocytes [3] and durable tumor response when it is treated with certain immunotherapy strategies [4]. On the other hand, the immunotherapy interest has increased since three new immunotherapy drugs for malignant melanoma have been approved by the FDA. These are an anti-CTLA4 antibody called ipilimumab (Yervoy®) and anti-PD1 antibodies called pembrolizumab (Keytruda®) and nivolumab (Opdivo®). However, only around 10-15% of advanced melanoma patients respond to these treatments. In this context, due to great advances in DNA technology and in knowledge of tumor cell-immune system interaction, gene therapy can be used to improve the antitumor immune response. In the field of immunotherapy, the different strategies of gene therapy are:

- Gene vaccines: DNA or RNA vaccines.
- Genetically modified cell vaccines: tumor cells or dendritic cells.
- Virotherapy: Genetically modified oncolytic virus.
- Gene silencing of immune checkpoint molecules.
- Genetically engineered T cells and chimeric antigen receptors (CAR)-T cell therapy.

Figure 1 shows these gene immunotherapy strategies in melanoma.

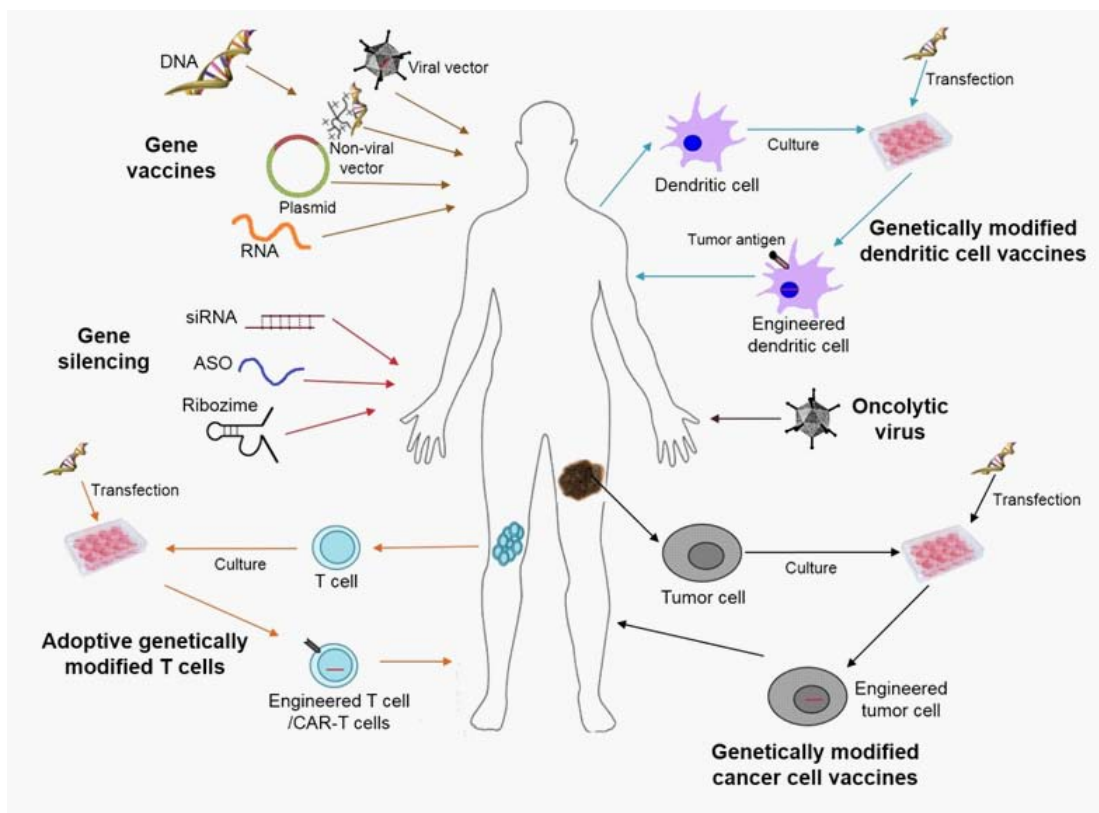


Figure 1: Strategies of gene immunotherapy in melanoma.

Genetic implementation allows introducing exogenous genes, such as tumor antigen or cytokine genes, into the cell for stimulating the antitumor immune response. The use of vectors facilitate DNA delivery into the cells. There are two kind of vectors: viral vectors (adenoviral, adenoassociated, retroviral, lentiviral, herpes simplex virus...) and non-viral vectors (cationic polymers, cationic lipids...). Viral vectors consist in viruses whose pathogenic genes have been removed and DNA of interest has been inserted in their genome. Normally, non-viral vectors are cationic molecules that bind DNA by electrostatic interactions with the anionic phosphate group of nucleotides. In general, viral vectors are more efficient than non-viral vectors but less safe. In addition, it is possible to avoid gene expression of certain immunosuppressive molecules involved in tumor tolerance through gene silencing. To silence genes different strategies exist. They are based in a short chain of nucleic acid which is complementary to a target mRNA or DNA. Three strategies are employed: small interfering RNA (siRNA), antisense oligonucleotides (ASO) and ribozymes. ASO technology has achieved a high development, synthesizing chemical modified ASOs that are resistant to nucleases. These ASOs can be administered to animals and humans without vectors, such as any other parenterally administered drug. Several targets of these immunotherapy strategies are represented in Figure 2.

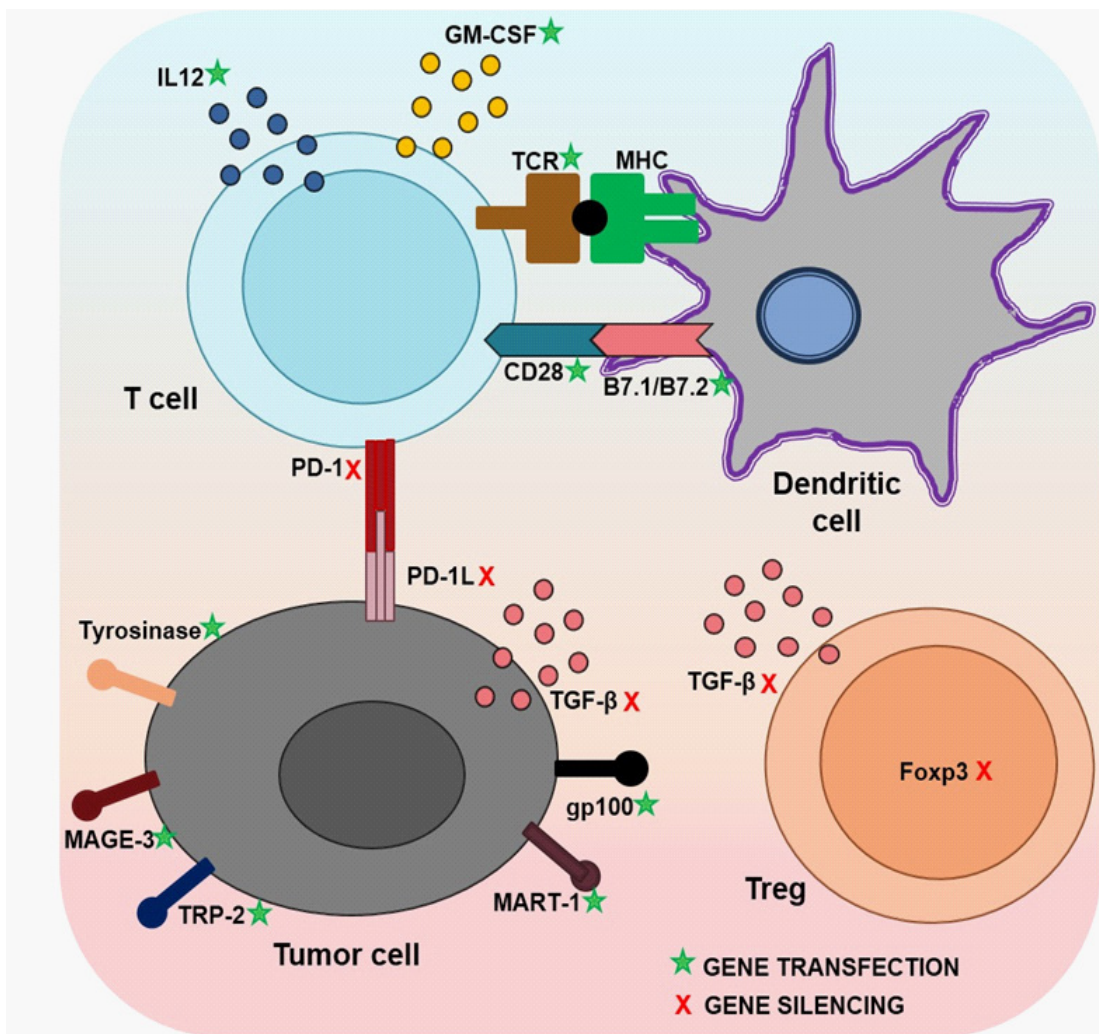


Figure 2: Targets for gene immunotherapy in melanoma.

In this chapter, the use of the different mentioned strategies of genetic immunotherapy for malignant melanoma are reviewed.

GENE VACCINES: DNA AND RNA VACCINES

DNA and RNA vaccines permit delivering multiple antigens in the same immunization and induce both humoral and cellular immune responses. These DNA and RNA encode tumor antigens or peptides. These vaccines have shown to be safe and tolerable [5-8]. Furthermore, they are cheaper than other vaccines. In general, non-viral delivery methods are preferred over viral vectors to transfect DNA or RNA due to their low cost, ease of large-scale production and, mainly, for their safety [9-11].

DNA Vaccines

Melanoma expresses several TAAs that can be encoded in DNA vaccines. Generally, the sequence of the gene that encodes the peptide is inserted in a plasmid. The DNA plasmid is administered to the patient and internalized by host cells, then transcribed in the nucleus and the resulting mRNA is released to the cytoplasm where it is finally translated. The proteasome processes the resulting polypeptides into peptides, which are ultimately presented on the surface of antigen presenting cells (APC) associated to major histocompatibility complex (MHC) molecules. This can occur directly by transfected APC or by cross presentation from non-APC to APC. The antigen bound to MHC complex is recognized by T cell receptors, inducing Ag-specific antibodies and cellular responses [12-15]. On the other hand, DNA vaccines are poorly immunogenic but their immunogenicity can be enhanced if the DNA contains unmethylated CpG motifs that stimulate the innate immune responses by interacting with Toll like receptor 9, expressed on the surface of APCs [16,17].

Several paths of DNA or RNA vaccine administration have demonstrated to trigger the antitumor immune response. Intramuscular injection of plasmid DNA reaches long term gene expression [18,19]. DNA delivery can be enhanced mediating electroporation. Electroporation transiently enhances cell membrane permeability, and by creates a low level of inflammation that recruits APCs to the injection site [20-22]. The efficacy of DNA vaccine is improved 100–1000 fold using electroporation [23]. Another strategy that has shown to increase the anti-melanoma immune responses of DNA vaccine is the use of nanoparticles to carry the DNA [24].

Both preclinical and clinical trials have assayed the efficacy and security of DNA vaccines. In a murine model, DNA vaccines encoding human gp100 (melanoma-associated antigen) induced cross-reactive and protective CD8⁺ T cell response against melanoma [25]. In other work that studied canine melanoma treatment, DNA vaccines expressing human tyrosinase induced the production of cross-reactive antibodies against canine and human tyrosinases and increased the animal half life [26,27]. These results suggest that a xenogeneic antigen is effective in inducing cross-reactive antitumor responses to self-antigen-expressing tumor cells. In a B16 model combining DNA vaccines coding for gp100 and Trp2 with anti-GITR antibodies, the treatment enhanced protection against B16 melanoma and induced CD8⁺ T cell memory responses [28]. Other study that combined DNA vaccines encoding Trp2 and gp100 with anti-CTLA-4 antibody demonstrated increased antigen specific T cell responses and anti-melanoma response, too [29]. CTLA4 is a T cell surface molecule that binds B7.1/B7.2 from the APC surface and triggers intracellular inhibitory signals mediating a negative regulation of the immune system. On the other hand, it has been demonstrated in mice that direct electroporation of IL-12 cDNA into tumors can induce tumor antigen specific cytotoxic lymphocyte responses achieving removal of established B16 melanoma [30,31]. In other preclinical study, vaccination with recombinant adenovirus vector expressing survivin (cancer over-expressed antigen) DNA and IL-2 cDNA, as adjuvant, reached effective antitumor responses in a murine melanoma model [32].

In a phase I clinical study carried out on 24 metastatic melanoma patients treated with intratumoral injection of IL-12 cDNA following electroporation, 10% (2/19) patients with non-electroporated distant lesions and no other systemic therapy showed complete regression of all metastases, whereas 42% (8/19) patients experimented disease stabilization or a partial response. These results suggest that intratumoral injection of IL-12 cDNA combined with electroporation can also be useful to treat cancer [33]. Only minimal systemic toxicity was reported. In other phase I clinical trial, patients with antigen positive solid tumors were vaccinated with intranodal delivery of a recombinant plasmid encoding fragments of PRAME (preferentially expressed antigen in melanoma) and PSMA (prostate-specific membrane antigen) and two peptides derived from them. In this study, specific T cell responses were observed in 63 melanoma patients and stable disease was achieved in 7 patients [34]. In another study with patients intranodally injected with DNA vaccines encoding tyrosinase epitope, antigen specific immune responses were induced in 42% (11/26) patients and overall survival was unexpectedly long, with 61% (16/26) patients alive at a median follow-up of 12 months [35]. However, in other clinical trial, patients with stage IV melanoma were intranodally injected with DNA vaccines encoding melanoma-associated antigen MART-1 (melan-A) but no clinical benefits were observed in spite of having induced antigen specific immune responses [36].

RNA Vaccines

It has been shown that RNA vaccines trigger higher immune response than DNA vaccines. After administration, mRNA is internalized by cells and is translated directly within the cytoplasm and then, the resulting peptides are presented to APC to stimulate an immune response [5,9]. Encoded products of mRNA are degraded by proteasomes and presented on MHC class I molecules to CD8⁺ T cells. They do not induce CD4⁺ T helper cell responses since there is not antigen presentation associated to MHC class II molecules. This MHC class II presentation can be achieved adding a lysosomal targeting signal to the antigen-encoding sequence triggering a CD4⁺ T cell response [37,38]. Furthermore, tumor antigen mRNAs fused to a signal peptide and a MHC class II sorting signal permits TAA presentation by both MHC class I and II molecules inducing CD4⁺ and CD8⁺ cell responses [39]. To enhance RNA immunogenicity its possible to provide costimulatory signals (for example CpG) that induce innate immune response via TLR, allowing the use of RNA as an adjuvant [40].

The use of naked mRNA as a vaccine is limited due to its rapid degradation by extracellular RNases [41,42]. One choice for DNA or RNA delivery is the use of gene gun. This delivery strategy has demonstrated to be effective for mRNA in animal models. In one model of mice B16 melanoma, administration of mRNA coding for the melanocyte self-antigen TRP2, linked to the immunogenic protein EGFP, using a gene gun induced antigen-specific cellular and humoral immunity and protected mice against melanoma lung metastases [43]. Another alternative to deliver mRNA is to condense it with protamine, a small arginine-rich polycationic protein, normally involved in DNA condensation [44]. This condensation with protamine improves the RNA stability. Furthermore,

protamine can trigger an innate immune response through MyD88, TLR7, and TLR8 dependent pathways [45-47]. In a phase I/II clinical study carried out in 21 patients with metastatic melanoma, intradermal injection of protamine-condensed mRNA encoding six melanoma-associated antigens proved to be safe, enhanced antigen-specific T cells in 50% (2/4) of evaluable patients, and achieved 14% (1/7) complete response (CR) rate in patients with measurable disease [48].

The DNA and RNA vaccines are a promising therapeutic alternative for malignant melanoma but further studies are needed combining DNA or mRNA vaccines with other therapeutic strategies to achieve better clinical outcomes.

GENETICALLY MODIFIED CELL VACCINES: TUMOR CELLS OR DENDRITIC CELLS

One of the most promising strategies of immunotherapy is vaccination with genetically modified cells. There are mainly two approaches with genetically modified cell vaccines: employing tumor cells or dendritic cells (DCs).

Tumor Cell Vaccines

It is possible to obtain tumor cells from the patients and then transfect them “ex vivo” with genes encoding immunoadjuvant molecules and finally re-infuse them into the same patient. Melanoma is a better candidate for this therapy compared to other types of cancer due to the facility to obtain tumor cells and its potential immunogenicity.

The efficacy of cancer vaccines can be improved by combining them with immunostimulatory molecules, such as IL-2, IL-15, IL-7, IFN and GM-CSF [49-52]. The immunoactivator molecule which has achieved the best results is probably GM-CSF [53]. Several studies in animal models show that the vaccination with sublethally irradiated genetically modified melanoma cells is able to induce an effective antitumor response, achieving the tumor growth suppression. In a preventive vaccination, these genetically modified cells have achieved the complete protection against tumor development in 100% of vaccinated animals [54-56]. However, in the therapeutic setting, vaccination with genetically modified cells delayed tumor growth and increased the half life of animals but finally the disease progressed and animals died [55-58]. Rarely, total tumor rejection is achieved in some animals employing therapeutic vaccination. So, in spite of the successful results achieved in preclinical trials their effectiveness in clinical trials is still poor.

The AGI-101H vaccine consists in 2 allogeneic melanoma cell lines, Mich-1 and Mich-2, transfected with cDNA encoding Hyper-IL-6 (H6) [59,60]. H6 is a protein composed by IL-6 fused with its agonistic soluble α receptor. AGI-101H was used in one study carried out in metastatic melanoma patients with high risk of disease recurrence and the estimated 5-year survival was 66.7%, 43.8% and 26.1% of patients with stages IIIB, IIIC and IV, respectively [61]. In other parallel study using this vaccine, the 5-year survival was seen in 56.3%, 39.8% and 41.2% of patients with stages IIIB, IIIC and IV, respectively. In patients not treated with surgery, disease control (CR, partial response, stable disease) was observed in 57% and 37% at these trials [61].

Treatment with AGI-101H showed to be safe. In most patients, the adverse events observed were mainly linked with local reactions at the injection site. The vaccine in a phase II trial with 77 patients with metastatic stage III and IV melanoma reached a median overall survival (OS) of 17.3 months. The estimated survival probability at 1-, 2-, 3-, 4-, 5-year was 64.9%, 35.1%, 20.8%, 14.3% and 13%, respectively. 19.4% and 9% of patients showed CR and partial response (PR), respectively. Disease control was reached in 54.5% of patients. The median CR+PR duration was 32 months. Reinduction was performed in 36.3% of patients following disease progression with 46.6% of CR+PR. No grade 3 or 4 toxicity was observed in this study [62].

Dendritic Cell Vaccines

Another approach of genetically modified cell vaccine employs dendritic cells. DCs are the most specialized APCs. In these vaccines, DCs are mainly transfected with genes of tumor peptides. In a preclinical study, vaccination with DCs transfected employing CL22, cationic peptide that condense DNA, to express the melanocyte differentiation antigen TRP-2, exerted protective effect against melanoma cell line B16F1 [63]. In a phase I/II trial on patients with metastatic melanoma, therapeutic vaccine consisting in DC transfected with a plasmid encoding the melanoma antigens melanA and gp100, employing CL22, demonstrated to be safe and well tolerated. The tumor response rate was 12%, with a further 16% of patients not progressing over the 12-week vaccination period [64]. In other clinical trial, patients with metastatic melanoma were vaccinated with mature DCs derived from monocytes, transfected with siRNA targeting the inducible proteasome and with RNAs encoding melanoma antigens MART1, MAGE-3, gp100, and tyrosinase. 50% (1/2) of patients with active disease had a PR, while the other, who suffered diffuse dermal and soft tissue metastases, achieved a CR [65].

VIROTHERAPY: GENETICALLY MODIFIED ONCOLYTIC VIRUSES

The oncolytic viruses are naive or genetically modified viruses that can only replicate in tumor cells. These viruses selectively destroy tumor cells. The use of oncolytic viruses genetically modified with immunoadjuvant molecules is other immunotherapy strategy that has reached promising results in melanoma. Normally, these viruses are genetically modified with some immunoadjuvant gene such as GM-CSF.

Currently, there are two oncolytic viruses approved, one in China and other in USA and in EU. China approved in 2006 the first oncolytic virus (H101, Oncorine®, modified adenovirus) for cancer therapy (neck squamous cell carcinoma). A clinical trial reported 79% objective response rate using H101 and chemotherapy compared with 40% reached with chemotherapy alone [66]. Then, T-VEC (Imlygic®, modified herpes simplex virus-1 expressing GM-CSF) was approved for use in advanced melanoma by the FDA in USA in 2015 and by the EMA in EU in December 2015.

There are several clinical studies that have shown antitumor clinical efficacy and safety using oncolytic viruses. A phase I clinical trial in patients with unresectable cutaneous melanoma previously vaccinated were treated with an oncolytic vaccinia virus genetically modified to

express GM-CSF (JX-594) reported 14% (1/7) CR, 42% (3/7) mixed response and 14% (1/7) PR [67]. In other phase I study on patients with unresectable melanoma, treatment with a vaccinia virus expressing B7.1 reported 16% (2/12) disease stabilization and 8% (1/12) objective CR [68]. Other study using vaccinia virus encoding three immunostimulatory molecules ICAM-1, B7.1 and LFA-3 (eV-TRICOM) showed 30% objective response [69]. In these studies only low grade toxicity was observed, restricted to the injection site.

Talimogene laherparepvec (T-VEC) [70], has demonstrated good results in clinical trials. A phase I study using T-VEC to treat solid tumor patients reported erythema, inflammation and pyrexia as the most frequent adverse effects [71]. In a phase II clinical trial, patients with stage IIIc or IV melanoma were treated with T-VEC and 26% (13/50) of overall objective response was observed [72]. In this study 16% (8/50) of patients experienced CR and 10% (5/50) PR. The one-year survival noted was 58% (29/50). A phase III clinical trial carried out in patients with unresectable melanoma compared treatment with T-VEC (295 patients) and treatment with GM-CSF (141 patients). Results reported 16.3% durable response rate (DRR) and 10.8% CR in patients treated with T-VEC and 2.1% DRR (and <1% CR) in patients treated with GM-CSF. In patients treated with T-VEC, the overall response rate was 26.4% and overall survival reported was of 23.3 months [73]. This study led to the T-VEC approval by the FDA and EMA.

It is possible that the combination of oncolytic viruses with other immunotherapy approaches increases the antitumor clinical response. The combination of T-VEC and ipilimumab for the treatment of melanoma in a phase I clinical trial only showed the expected side effects and reported around 56% (50/83) objective response rate [74].

GENE SILENCING OF IMMUNE CHECKPOINT MOLECULES

The approval by the FDA of ipilimumab in 2011 (anti-CTLA4 antibody) and pembrolizumab and nivolumab (anti-PD1 antibodies) in 2014 for the treatment of patients with advanced metastatic melanoma has increased the confidence in blockade of immunosuppressive molecules as a useful therapy against a range of tumors. However, there are some molecules such as nuclear transcription factor Foxp3, that have a role in the failure of the antitumor immune response, which cannot be blocked by antibodies due to their intracellular location. Gene silencing permits blocking these intracellular molecules and presents an alternative mechanism to tie up the expression of CTLA4, PD1 and other immunosuppressive molecules.

This year it has been published a preclinical study in a model of murine melanoma using a plasmid encoding siRNA to silence Foxp3. In this study, the Foxp3 silencing achieved the tumor growth delay and the modification of tumor immunosuppressive environment [75].

It is known that the failure of antitumor response employing cell vaccines in therapeutic setting is due to the presence of negative immunoregulatory molecules. Thus, the combination of vaccination and gene silencing of immunosuppressive molecules is presented as a promising strategy for cancer treatment.

A preclinical study in a model of B16 mouse melanoma using TGF- β 1 siRNA and a TLR-activated antigen-pulsed dendritic cell vaccine in a therapeutic setting showed a tumor growth delay [76].

A phase I clinical trial employing autologous tumor cells transfected with a plasmid encoding TGF- β antisense oligonucleotide (ASO) and GM-CSF gene (TAG vaccine) proved its safety and tolerability. In this study, the 1 year-survival reported was 35% (7/20) of patients [77]. A CR of all target and non-target lesions was observed in one patient with metastatic melanoma who had previously failed standard therapy.

The combination of immunosuppressive molecules with another immunotherapy strategies is an interesting approach that should be studied in future clinical trials.

GENETICALLY ENGINEERED T CELLS AND CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL THERAPY

Due to the immunogenic potential of melanoma and relative facility to obtain tumor infiltrated lymphocytes (TILs), adoptive tumor-specific T cell therapy has been tested almost exclusively to treat patients with advanced melanoma. However, sometimes it is difficult or impossible to obtain TILs. In these cases, one choice is to obtain peripheral blood lymphocytes from patients, then transfect them "*ex vivo*" with the gene of a T cell receptor engineered to specifically recognize certain TAA and finally inject them into the same patient. T cells genetically modified to display TAA-specific TCRs have proved activity against several kind of human cancer cells and succeeded in achieving tumor regression in patients with metastatic melanoma [78-82]. In patients with advanced melanoma, treatment with T cells engineered to express specific TAA-T receptor against MART-1 reached better responses [83,84].

In a recent study using lymphocytes genetically modified to express a MART-1-specific TCR, tumor regression was observed in 30% (6/20) of metastatic melanoma patients [85]. Severe toxicity in ears, eyes or skin of patients treated with lymphocytes with MART-1- or gp100-specific TCR was observed due to an autoimmune response against melanocytes producing histological destruction in these healthy tissues [81]. In several clinical trials using MAGE-A3-specific high-affinity TCR, effectiveness has been correlated with appearance of adverse events in brain and heart. In a trial, 33% (3/9) of patients treated with MAGE-A3-specific high-affinity TCR (HLA-A2-restricted) exhibited mental disturbance, and two of them experienced leukoencephalopathy and died [84]. In other study employing another MAGE-A3-specific high-affinity TCR, two patients suffered a cardiac shock and died [85]. Other trial using T cells transduced with a TCR targeting NY-ESO-1, reported objective clinical responses in 45% (5/11) of patients with melanoma [86]. In another clinical trial, objective clinical responses were reported in 55% (11/20) of NY-ESO-1 melanoma patients treated with autologous T cells retrovirally transduced with an NY-ESO-1-reactive TCR. In this study, the estimated overall 3-year and 5-year survival rates were both of 33% [87].

The second approach for retargeting of T cells is to introduce a completely engineered antigen receptor also called chimeric antigen receptor (CAR). The CAR has been designed to effectively combine the high-affinity antigen recognition domain of a single chain variable fragment (scFv) of monoclonal antibody (mAb) with the efficient killing machinery of a T cell. This was accomplished by creating a molecule that links the variable domains of an antibody to the intracellular signaling domains of the TCR complex. Thus, the recognition afforded by CAR is MHC-independent and is directed to a protein expressed on the surface of tumor cells. This is the main advantage of the recombinant CARs over the transduced TCRs. The first CAR generation consisted in a fused protein composed by scFv and the signal transduction domain of CD3 ζ separated by a spacer region. The second CAR generation maintains the structure of the first generation but incorporates an intracellular fragment of a costimulatory molecule (CD28). Third generation CAR incorporates two costimulatory molecules (CD28 and 4-1BB). Lymphocytes are transfected “*ex vivo*” to express CAR and then they are administered to the patient.

CAR T cells have reached great successful in hematological malignances, in particular in acute lymphoblastic leukemia [88,89]. They also have been proved in some solid tumors but the efficacy is lower than in hematological malignances [90,91]. Currently, there is not any clinical trial using CAR in melanoma, probably due to the good results achieved using TILs, reaching 49–72% objective clinical response(CR + PR) in metastatic melanoma patients with previous treatments [92,93].

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