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Antigen-specific vaccines for cancer treatment

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Abbreviations: MHC, major histocompatibility complex; BCG, Bacille Calmette-Guerin; GM-CSF, granulocyte macrophage-colony stimulating factor; DCs, dendritic cells; APCs, antigen-presenting cell; NSCLC, non-small-cell lung carcinoma; TAAs, tumor-associated antigens; MAGE-A1, Melanoma-associated antigen 1; CT, Cancer-testis; SSX-2, Synovial sarcoma X breakpoint 2; PSA, Prostate-specific antigen; hTERT, human Telomerase reverse transcriptase; TACAs, Tumor-associated carbohydrate antigens; WGS, whole genome sequencing; WES, whole exome sequencing; HLA, human leukocyte antigen; Ig Id, immunoglobulin idiotype; BCR, B-cell receptor; TPA, transporter associated with antigen processing; MS, mass spectrometry; GB, glioblastoma; RCR, renal cell cancer; CRC, colorectal cancer; FDA, Food & drug administration; TLRs, Toll-Like Receptors; HER2, human epidermal growth factor receptor 2; PRRs, Pattern Recognition Receptors; HSPs, stress/heat shock proteins; TARP, T-cell receptor gamma alternate reading frame protein; LPs, long peptides; CTL, cytotoxic T-lympocites; IFNg, interferon gamma; HPV, human papillomavirus; CDCA1, cell division cycle associated 1; PAP, prostatic acid phosphatase; mCRPC, metastatic castrate-resistant prostate cancer; EGT, electro-gene-transfer; MVA, modified vaccinia strain Ankara

Vaccines targeting pathogens are generally effective and protective because based on foreign *non-self* antigens which are extremely potent in eliciting an immune response. On the contrary, efficacy of therapeutic cancer vaccines is still disappointing. One of the major reasons for such poor outcome, among others, is the difficulty of identifying tumor-specific target antigens which should be unique to the tumors or, at least, overexpressed on the tumors as compared to normal cells. Indeed, this is the only option to overcome the peripheral immune tolerance and elicit a non toxic immune response. New and more potent strategies are now available to identify specific tumor-associated antigens for development of cancer vaccine approaches aiming at eliciting targeted anti-tumor cellular responses. In the last years this aspect has been addressed and many therapeutic vaccination strategies based on either whole tumor cells or specific antigens have been and are being currently evaluated in clinical trials. This review summarizes the current state of cancer vaccines, mainly focusing on antigen-specific approaches.

Tumor Cell Vaccines

Tumors accumulate several genetic modifications in somatic cells[1,2] which provide selective growth advantage to cancer cells in order to initiate clonal expansion.[3]

Considering the high number of potential tumor antigens for each individual cancer, vaccination with whole tumor cells has been considered the optimal strategy to include all potentially relevant antigens. Moreover, such vaccine approach circumvents the major histocompatibility complex (MHC)- restriction and the need for specific patient-tailored epitope identification.

Autologous tumor vaccines prepared using patient-derived tumor cells represent one of the first types of cancer vaccines that have been tested.[4] The efficacy of such approach has been evaluated during the years in several clinical trials targeting different tumor types, including lung cancer,[5,6] colorectal cancer,[7-9] melanoma,[10-12] renal cell cancer[13,14] and prostate cancer.[15] However, sufficient amount of tumor specimen is needed for preparation of such autologous tumor cell vaccines, restraining its application to a limited number of tumor types or stages.

To overcome the limitations of patient-tailored vaccines, allogeneic whole tumor cell vaccines have been developed based on 2 or 3 established human tumor cell lines. In particular, they allow standardization of large-scale production, quality and composition of the vaccines as well as comparative analysis of clinical outcome. Moreover, they can be easily manipulated for expression of immunostimulatory molecules.

The first allogeneic whole-cell vaccine was the Canvaxin™, consisting of 3 melanoma lines combined with BCG as an adjuvant[16] which, after promising results in phase II clinical trials,[17,18] failed in 2 multi-institutional randomized phase III trials.[19]

However, the effectiveness of such vaccine strategy is dramatically hampered by the immune system's inherent tolerance to several antigens expressed in the whole tumor cell preparation, as they may be expressed by normal tissues or presented to T cells in a non-stimulatory context. In order to break tolerance and contain immune suppression, antigens should be combined to strong immunological adjuvants (reviewed in[20,21]). To this aim, whole tumor cell vaccines (autologous or allogeneic) can be genetically modified to express co-stimulatory molecules and/or cytokines, such as granulocyte macrophage-colony stimulating factor - GM-CSF (GVAX). GVAX has proven to be more effective than others in inducing recruitment, maturation, and

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function of dendritic cells (DCs), the most potent antigen-presenting cell (APC).[22–24]

The clinical activity of GVAX based on allogeneic whole-cell vaccine has been evaluated for treatment of recurrent prostate cancer,[25,26] breast cancer[27] and pancreatic cancer.[28] However, the use of allogeneic cells as a vaccine can generate strong anti-MHC immune reactions that can interfere with the anti-tumor response and recent observations suggest a potential detrimental effect of GM-CSF due to induction of immune suppression in cancer patients (reviewed in[29,30]).

An alternative strategy to improve immunogenicity of allogeneic tumor cell vaccines is to engineer cell lines to secret antisense oligonucleotide for inhibiting expression of the immunosuppressive cytokine TGF-β2. This strategy is the principle of Lucanix™, targeting non-small-cell lung carcinoma – NSCLC, which in 2 independent phase 2 trials has induced significant improvement in overall survival in advanced disease.[31,32] The phase 3 STOP (Survival, Tumor-free, Overall, and Progression-free) trial is in progress enrolling patients with locally advanced or advanced NSCLC without progression after first-line chemotherapy or chemoradiation (NCT00676507).

Tumor-Associated Antigens - TAAs

Shared TAAs

Cancer vaccines based on defined specific tumor antigens should elicit a very specific effector and memory cell response. However, such approach may result in selection and expansion of tumor variants which lack the target tumor antigen and are resistant to the vaccine-induced immune response. Nevertheless, the newly expressed antigens on tumor variants may elicit a broader anti-tumor immune response, in a process defined "epitope spreading."[33,34]

MAGE-1 was the first gene reported to encode a human tumor antigen recognized by T cells.[35] Since then, a large number of tumor-associated antigens (TAAs) have been described and are divided into ***shared*** and ***unique*** TAAs.[36] A complete and update list of shared TAAs is available at http://www.cancerimmunity.org/peptide/.

Shared TAAs can be classified in 3 main groups: 1) cancer-testis; 2) tissue differentiation; and 3) widely occurring overexpressed antigens. Cancer-testis (CT) antigens result from reactivation of genes which are normally silent in adult tissues,[37] but are transcriptionally activated in different tumor histotypes.[38] Many CT antigens have been identified and tested in clinical trials, although little is known about their specific functions, especially with regards to malignant transformation. Such group of TAAs includes the MAGE-A1,[39,40] NY-ESO-1[41] and SSX-2.[42] Tissue differentiation antigens are shared between tumors and the normal tissue of origin; they are mostly found in melanomas and normal melanocytes (Gp100, Melan-A/Mart-1, Tyrosinase).[43-48] as well as in epithelial tissues and tumors such as prostate (PSA)[49,50] and breast carcinomas (Mammaglobin-A).[51] Widely occurring overexpressed TAAs are over-expressed in tumor cells compared to normal tissues, reaching the threshold for T cell recognition to break the immunological tolerance and trigger an anticancer response. The antiapoptotic proteins livin and survivin,[52,53] hTERT,[54-56] and tumor suppressor proteins (e.g., p53)[57,58] belong to such group. Mucin 1 (MUC1) belongs to the "overexpressed TAA" category, although it is the combination of overexpression and modification of glycosylation status in tumor cells to make MUC1 highly immunogenic and, thus, an interesting target in cancer immunotherapy.[59]

Tumor-associated carbohydrate antigens (TACAs) represent an additional class of shared tumor antigens. They are glycans uniquely or overexpressed by tumors[60] correlating also with various stages of cancer development.[61,62]

Unique personalized TAAs

Unique TAAs result from random somatic point mutations induced by physical or chemical carcinogens, and therefore represent neo-antigens uniquely expressed by individual tumors (reviewed in[63,64]). Cancer genome instability and subsequent selective pressure lead to accumulation of mutations which may give rise to non-synonymous mutations. Interestingly, the number of such non-synonymous mutations shows a significant variability between different tumor types (10 to 400).[2,65] Given that neo-antigens are tumor – specific, their immunogenicity is not hampered by central T-cell tolerance and the elicited T-cell responses are not expected to result in autoimmune toxicity. Indeed, mutated epitopes identified in a murine melanoma cells have been shown to elicit a stronger T-cell response *in vivo* in a side-by-side comparison with corresponding wild type epitopes.[66] Moreover, neo-antigens should be more resistant to immune-selection being crucial to the oncogenic process and thus indispensable for maintaining the neoplastic state. Most of the studies focused on cancer mutation discovery have been performed using broad assays like whole genome (WGS) and whole exome sequencing (WES) on each individual tumor,[67,68] in order to identify mutated genes and select peptides whose motifs are predicted to be presented by the patient's HLA alleles. However, only a small fraction of such mutated peptides are indeed presented by MHC or recognized by T cells, and this seems to directly correlate with the tumor-specific mutation load.[66,69-72] Therefore, prediction of MHC presentation calculated by software algorithms needs to be confirmed by experimental procedures. Moreover, each tumor bears highly heterogeneous sets of defects in dozens of different genes[73-77] which need to be further verified for their substantial contribution to the tumor development and progression and, consequently, for their relevance as vaccine target.[78]

On the contrary, identification of unique TAAs for hematological tumors as B cell lymphomas requires sequencing analysis focused only on immunoglobulin idiotype (Ig Id) included in the B-cell receptor (BCR), which represents the target antigen.[79,80]

Selection of antigens for cancer vaccine development

TAAs may be used as vaccine administering the full-length protein, which contains all potential MHC class I and MHC class II epitopes capable of stimulating CD8+ and CD4+ T

cells, respectively. Therefore, the full-length protein can be considered as an "off-the-shelf" vaccine ready-to-use for any eligible cancer patient ***regardless*** his/her HLA allele background. On the contrary, vaccines based on epitopes derived from TAAs require the identification and selection of specific epitopes that interact with specific MHC complexes in order to stimulate a T-cell-associated immune response. Such epitopes represent an "off-the-shelf" vaccine ready-to-use for any eligible cancer patient ***characterized by*** that specific HLA allele background. In the last years, this has been performed by predictive immune-informatics algorithms.[81-85] Prediction algorithms have been constantly updated in order to take into considerations all the biological variables related to the complexity of the intra-cellular process governing the peptide fragmentation by the proteasome and the transportation to HLA class I molecules in the endoplasmic reticulum, via the transporter associated with antigen processing (TAP) (http://www.cbs.dtu.dk/services/). Nevertheless, immunological experimental validation of predicted epitopes is required to ultimately confirm the selection of epitopes.

Recently, strategies based on high resolution mass spectrometry (MS) have been developed for directly sequencing peptides presented by HLA molecules (***HLA ligandome***) on tumor cells, to identify naturally processed class I and class II tumor-associated peptides.[86] This strategy, indeed, allows the identification of T cell epitopes in fact presented by the tumor cells, thus representing a valid target of the T cells, and it has been employed to identify the HLA ligandome for glioblastoma (GB),[87] renal cell cancer (RCC) and colorectal cancer (CRC) (reviewed in[88]).

In the quest of the most specific tumor-associated antigens, a personalized approach is currently feasible based on the individual features of tumors. Next-generation sequencing and computation prediction allow the identification of genetic alterations in cancer cells of each cancer patient (the mutanome) encoding unique mutated peptides (m-peptides) that can be used as vaccine to elicit specific anti-tumor T cells.[66,89] The latter approaches represent the very last frontier of the immunotherapy and their translation into clinical application is currently used in 2 projects funded by the European Union, within the Framework Program 7, focused on glioma (www.gapvac.eu) and on hepatocellular carcinoma (www.hepavac.eu).

Peptide-protein based cancer vaccines

Peptide-protein based vaccines are cost effective, compared to other vaccine approaches including multiple antigens. For such reason, most of cancer vaccine clinical trials have been performed with peptide-protein based vaccines including cancer-testis or differentiation TAAs but, despite the induction of strong T-cell immunity, clinical outcomes have been disappointingly limited[90-95] (**Table 1 and 2**).

Indeed, with exception of the 2 cancer vaccine clinical trials based on Sipuleucel-T which have allowed the licensing by FDA for the treatment of asymptomatic metastatic castrate-resistant prostate cancer (see below),[96,97] the other 8 Phase 3 clinical trials completed or terminated have not provided satisfactory results and no further implementation for licensing has been pursued (**Table 3**).

Among many possible reasons for such unsatisfactory results, one could be the induction of a restricted T cell immune response that may not be sufficient and ultimately cause a selection of tumor cells lacking or down-regulating the targeted antigen. The use of multiple peptides derived from different TAAs could overcome such a drawback, eliciting a T cell response against multiple targets which may counteract tumor heterogeneity and enhance the probability of tumor eradication. The feasibility of such multi-epitope approach has been confirmed by *in vivo* and *in vitro* studies showing that multiple peptides do not mutually compete for MHC presentation and are able to induce a multi-specific T-cell response.[98-100] Furthermore, studies have also clearly demonstrated that a potent and sustained CD8+ T-cell response can be induced only combining HLA class I and II-restricted peptides, due to the helper function provided by CD4+ T helper (TH) cells.[101-103] Vaccines based on a multi-peptide cocktail have been developed and evaluated in phase I/II clinical trials for glioblastoma (IMA950, NCT01920191), renal cell carcinoma (IMA901, NCT00523159) and colo-rectal cancer (IMA910, NCT00785122) showing feasibility, safety and immunogenicity. IMA901 is currently in a world-wide phase 3 trial in patients receiving Sunitinib for advanced/metastatic RCC (NCT01265901).

Strategies to improve immunogenicity of peptide-based vaccines

Several strategies have been adopted to improve clinical outcome of peptide-based vaccines, mainly aiming at potentiating the innate immune response. Toll-Like Receptors (TLRs) agonists are being tested in clinical trials evaluating peptide/protein-based cancer vaccines. TLR3 agonists currently evaluated in human clinical trials are the poly(I) poly(C12U) (Ampligen®), in a phase I-II study of HER2 vaccination in breast cancer patients (NCT01355393) and the Poly-ICLC (Hiltonol®) in a multipeptide vaccine in melanoma patients (NCT01585350), in a MAGE-A3 ASCI peptide vaccine in melanoma patients (NCT01437605) as well as in a MUC1 peptide vaccine in patients with advanced colorectal adenoma (NCT00773097). The TLR7/8 agonist Resiquimod is currently evaluated in a gp100(g209–2M) and MAGE-3 peptide vaccine in patients with melanoma (NCT00960752). Additional agonists for Pattern Recognition Receptors (PRRs) are evaluated for their adjuvant activity in therapeutic cancer vaccines. In particular, stress/heat shock proteins (HSPs) can be utilized as immunostimulatory agents for cancer immunotherapy.[104–106] Chaperoning technology has been generated to formulate recombinant HSP vaccines including clinically relevant tumor antigens (e.g.,, gp100, HER-2/Neu) (reviewed in[107]). Such strategy may be used to develop many different antigen targets[108] and 2 phase I clinical trials of recombinant chaperone vaccine targeting melanoma have been designed, one completed (NCT00005633) and one currently recruiting patients (NCT01744171).

A phase III clinical trial has shown that melanoma patients in the M1a and M1b substages, receiving a larger number of immunizations with vitespen (autologous, tumor- derived heat shock

Table 1. Cancer vaccines in Phase 1/2 clinical trials based on peptide/protein strategies.

CANCER	Antigen	STRATEGY	NCT NUMBER	PHASE
Bile duct	URLC10	Peptide	NCT00624182	Phase 1
Bladder	NY-ESO-1	Peptide	NCT00070070	Phase 1
Brain	GAA	DC	NCT00612001	Phase 1
Breast	OFA	DC	NCT00715832	Phase 1
	cyclin B1/WT-1/CEF	DC	NCT02018458	Phase 1/2
	VEGFR1 and VEGFR2	Peptide	NCT00677326	Phase 1/2
	TTK	Peptide	NCT00678509	Phase 1/2
	Multiple	Peptide	NCT00674791	Phase 1
	MUC1-KLH	Protein	NCT00004156	Phase 1
	OFA	DC	NCT00879489	Phase 1/2
	HER2	Protein	NCT00952692	Phase 1/2
Cervical	HPV16 E7	DC	NCT00155766	Phase 1
Colorectal	CEA	DC	NCT00228189	Phase 1/2
	VEGFR1 and VEGFR2	Peptide	NCT00677612	Phase 1/2
	Multiple	Peptide	NCT00677287	Phase 1/2
	Multiple IMA910	Peptide	NCT00785122	Phase 1/2
Esophageal	URLC10	Peptide	NCT00753844	Phase 1
	URLC10, VEGFR1 and VEGFR2	Peptide	NCT00681421	Phase 1/2
	URC10, TTK, KOC1	Peptide	NCT00681330	Phase 1/2
	Multiple	Peptide	NCT00669292	Phase 1/2
Gastric	URLC10	Peptide	NCT00845611	Phase 1/2
	URLC10, VEGFR1 and VEGFR2	Peptide	NCT00681252	Phase 1/2
	URLC10, KOC1, VEGFR1 and VEGFR2	Peptide	NCT00681577	Phase 1/2
Gliobalstoma	not specified	DC	NCT00576641	Phase 1
	not specified	Peptide	NCT01854099	Phase 1
	SL-701	Peptide	NCT02078648	Phase 1/2
	Multiple IMA950	Peptide	NCT01403285	Phase 1
	Multiple IMA950	Peptide	NCT01920191	Phase 1/2
	Multiple	Peptide	NCT02149225	Phase 1
Hematological	WT1	Peptide	NCT00672152	Phase 1
Leukemia	WT1	DC	NCT00923910	Phase 1/2
Melanoma	p53; survivin; telomerase	DC	NCT00197912	Phase 1/2
	MART-1; gp100; tyrosinase	Peptide	NCT00005841	Phase 1
	MART-1; gp100; Tyrosinase; NY-ESO-1	DC	NCT00313508	Phase 1
	NY-ESO-1	Protein	NCT01079741	Phase 1/2
	GSK2302025A	Protein	NCT01149343	Phase 1
	MART-1, gp100; tyrosinase	Peptide	NCT00028431	Phase 1
	gp100; tyrosinase	DC	NCT01530698	Phase 1/2
	tyrosinase	Protein	NCT01331915	Phase 1/2
	Multiple	DC	NCT00124124	Phase 1
	gp100	Peptide	NCT00003229	Phase 1/2
	MART-1; MAGE-3.1; survivin	DC	NCT00074230	Phase 1/2
	MART-1, gp100	Peptide	NCT00470015	Phase 1
	MART-1; MAGE-3.1	Peptide	NCT00002952	Phase 1/2
	MART-1, gp100; tyrosinase	DC	NCT00003665	Phase 1
	MAGE-10.A2; MART-1; NY-ESO-1; tyrosinase	Peptide	NCT00037037	Phase 1
	MART-1; gp100	Peptide	NCT00091338	Phase 1
	gp100	Peptide	NCT00091143	Phase 1
	MART-1; gp100	Peptide	NCT00019214	Phase 1/2
	MART-1; gp100	Peptide	NCT00010309	Phase 1/2
	OVA BiP; gp209–2M; tyrosinase peptide	Peptide	NCT00005633	Phase 1
	MART-1; gp100; MAGE-3.1; tyrosinase	Peptide	NCT00003792	Phase 1
	gp100; MART-1	Peptide	NCT00004025	Phase 1/2
	gp100	Peptide	NCT00003897	Phase 1
	MAGE-1/MAGE-3; tyrosinase; MART-1; gp100	DC	NCT01082198	Phase 1/2
	Melan-A	Peptide	NCT00324623	Phase 1
	MART-1; NY-ESO-1; gp100	Peptide	NCT01176461	Phase 1
	gp100(g209–2M)	Peptide	NCT00960752	Phase 2
	gp100; tyrosinase	DC	NCT00243529	Phase 1/2
	MAGE-3.A1; NA17.A2	Peptide	NCT01191034	Phase 1/2
Multiple	KOC1, TTK, CO16, DEPDC1, MPHOSPH1	Peptide	NCT00676949	Phase 1

(*continued on next page*)

Table 1. Cancer vaccines in Phase 1/2 clinical trials based on peptide/protein strategies. *(Continued)*

CANCER	Antigen	STRATEGY	NCT NUMBER	PHASE
	HER2, NY-ESO-1	Peptide	NCT00291473	Phase 1
	MAGE-12	Peptide	NCT00020267	Phase 1
	NY-ESO-1	Peptide	NCT01584115	Phase 1/2
	ONT-10	glycolipopeptide	NCT01556789	Phase 1
	ONT-10	glycolipopeptide	NCT01978964	Phase 1
	CEA	Peptide	NCT00057915	Phase 1
Neuroblastoma	GD2L and GD3L	Protein	NCT00911560	Phase 1/2
Non Small Cell Lung	GSK2302032A	Protein	NCT01159964	Phase 1
	URLC10; CDCA1; VEGFR1; VEGFR2	Peptide	NCT00874588	Phase 1
	URLC10; TTK; KOC1	Peptide	NCT00674258	Phase 1/2
	URLC10; VEGFR1; VEGFR2	Peptide	NCT00673777	Phase 1/2
Ovarian	Survivin	Peptide	NCT01416038	Phase 1/2
	Multiple	Peptide	NCT01095848	Phase 1
Pancreatic	MUC1	Peptide	NCT00008099	Phase 1
Prostate	TF	Protein	NCT00003819	Phase 1
	rsPSMA	Protein	NCT00705835	Phase 1
	PSA	DC	NCT00005992	Phase 1
	MUC-2	Protein	NCT00004929	Phase 1
	MUC-2	Protein	NCT00036933	Phase 1
	PSA; PAP; KLH	DC	NCT01171729	Phase 1/2
Renal cell	Survivin; TERT	DC	NCT00197860	Phase 1/2
Sarcoma	NY-ESO-1; MAGE-A1; MAGE-A3	DC	NCT01241162	Phase 1
	NY-ESO-1; MAGE-A1; MAGE-A3	DC	NCT00944580	Phase 1
	NY-ESO-1	Peptide	NCT00027911	Phase 1

protein gp96 peptide complexes), have a longer survival than those receiving fewer such treatments.[109]

Additional strategies to improve immunogenicity of peptides aims to generate peptide variants of TAAs, including mimotopes, heteroclitic peptides, altered-peptide ligands (reviewed in[110]) as well as introducing amino acid substitutions in the peptide-MHC-binding surface.[111-113] A clinical trial based on a novel prostate and breast cancer antigen TARP, designed as an "epitope-enhanced" or "anchor-modified" peptide,[114] is currently conducted in stage D0 prostate cancer patients (NCT00972309) with promising early clinical results.[115] In addition, the above mentioned gp100(g209–2M) peptide evaluated in a clinical trial in melanoma patients (NCT00960752) is, indeed, an "epitope-enhanced" or "anchor-modified" peptide.[116]

Furthermore, long peptides (LPs) have been shown to be more immunogenic than individual MHC class I-restricted short peptide.[117] Indeed, LPs do not bind directly to MHC class I but only through processing by DCs,[118-120] resulting in a significant reduction of transient CTL response or tolerance.[121,122] Moreover, LPs may persist longer in inflamed lymph nodes sustaining the clonal expansion of IFNg-producing effector T cells with improved anti-tumor CTL response.[118] LPs have been generated linking CTL and Th epitopes, as shown for several TAAs including human papillomavirus (HPV) E6-E7 antigens,[123,124] the CT antigen NY-ESO-1[125] and HER-2/neu[126,127] and, very recently, the novel cancer-testis antigen, cell division cycle associated 1 (CDCA1).[128] Five clinical trials have been designed using LPs, 2 targeting melanoma based on NY-ESO-1 (NCT00112242) and on multiple TAAs (NCT02126579), 2 targeting ovarian cancer based on p53 TAA (NCT00844506 and NCT01639885) and one targeting cervical cancer based on HPV E6/E7 proteins (NCT02128126).

In the last years, it has been shown that the blockade of immune checkpoints by antibodies or modulated by recombinant forms of ligands or receptors (such as MAbs to PD-1, PDL-1, CTLA4) represents one of the most promising approaches to improve therapeutic antitumour immunity, amplifying antigen-specific T-cell responses.[129] Therefore, the combination of a vaccine and blockade of immune checkpoints could result in elicitation of a stronger immune response with a more potent control of tumor growth. A clinical trial of patients with advanced melanoma evaluated the effect of a peptide vaccine of melanoma-specific gp100 combined with humanized CTLA4 antibody ipilimumab, showing a 3.5 month survival benefit compared with the group receiving the gp100 peptide vaccine alone.[130] Few clinical trials have been or are currently conducted to investigate the combinatorial effect of TAA-based cancer vaccine and ipilimumab in patients with melanoma (MART-1 - NCT00090896; gp100 - NCT00094653; Tyrosinase/gp100/MART-1 - NCT00025181) or pancreatic cancer (PSA - NCT00113984). Similarly, few clinical trials are currently conducted to investigate the combinatorial effect of TAA-based cancer vaccine and anti-PD-1 antibody BMS-936558 in patients with melanoma (multiple epitopes - NCT01176461 and NCT01176474).

Dendritic Cells as Antigen Delivery System

Increased immunogenicity of peptides for cancer vaccine can be achieved by loading autologous dendritic cells (DCs) either *ex vivo* or *in vivo* with the peptide.[131-133] Indeed, DCs are the professional antigen-presenting cells (APCs) bridging innate and adaptive immunity.[134] Their role in the periphery is to uptake

Table 2. Cancer vaccines in Phase 2 or 3 clinical trials based on peptide/protein strategies.

CANCER	Antigen	STRATEGY	NCT NUMBER	PHASE
Bladder	MPHOSPH1 and DEPDC1	Peptide	NCT00633204	Phase 2
Breast	MUC1	Peptide	NCT00925548	Phase 3
Cervical	HPV16/18	Protein	NCT01356823	Phase 2
	HPV16/18	Protein	NCT01735006	Phase 3
Colorectal	not specified	DC	NCT01348256	Phase 2
	not specified	DC	NCT01413295	Phase 2
Esophageal	STF-II	Peptide	NCT01267578	Phase 2
	G17DT	Peptide	NCT00020787	Phase 3
Glioblastoma	ICT-107	DC	NCT01280552	Phase 2
Hodgkin/Non-Hodgkin	LMP2A	DC	NCT02115126	Phase 2
Melanoma	tyrosinase	Peptide	NCT01989572	Phase 3
	gp100; tyrosinase; MAGE-3.1	Peptide	NCT00085189	Phase 2
	gp100; tyrosinase; MART-1	Peptide	NCT00089063	Phase 2
	MART-1; NA17-A; gp100; tyrosinase	Peptide	NCT00036816	Phase 3
	MART-1; gp100; tyrosinase	Peptide	NCT00031733	Phase 2
	gp100; tyrosinase	Peptide	NCT00003339	Phase 2
	MART-1; gp100; tyrosinase	DC	NCT00334776	Phase 2
	gp100	Peptide	NCT00032045	Phase 2
	MART-1; gp100; tyrosinase	Peptide	NCT00019396	Phase 2
	MART-1; gp100	Peptide	NCT00295958	Phase 2
	MART-1, gp100 and tyrosinase	Peptide	NCT00001685	Phase 2
	MART-1; gp100	Peptide	NCT00020475	Phase 2
	MART-1, gp100; tyrosinase	Peptide	NCT00059475	Phase 2
	gp100 antigen	Peptide	NCT00080353	Phase 2
	MART-1, gp100; tyrosinase	Peptide	NCT00006113	Phase 2
	MART-1, gp100; tyrosinase	Peptide	NCT00006385	Phase 2
	MART-1; gp100	Peptide	NCT00019721	Phase 2
	MART-1; gp100	Peptide	NCT00019994	Phase 2
	gp100	Peptide	NCT00072085	Phase 2
	gp100; tyrosinase	Peptide	NCT00003222	Phase 2
	gp100; tyrosinase	Peptide	NCT00003362	Phase 2
	gp100; tyrosinase	Peptide	NCT00003274	Phase 2
	gp100	Peptide	NCT00003568	Phase 2
	multi-epitope	Peptide	NCT00071981	Phase 2
	gp100; tyrosinase	Peptide	NCT00020358	Phase 2
	gp209–2M	Peptide	NCT00019487	Phase 2
	NY-ESO-1	Peptide	NCT00079144	Phase 2
	multi-epitope	Peptide	NCT00004104	Phase 2
	gp100	Peptide	NCT00077532	Phase 2
	NY-ESO-1	Peptide	NCT00020397	Phase 2
	gp100	Peptide	NCT00019682	Phase 3
	gp100; MART-1	Peptide	NCT00303836	Phase 2
	NA17.A2; MAGE-3.1; MART-1	Peptide	NCT01307618	Phase 2
	gp100; MART-1	DC	NCT00019890	Phase 2
Multiple	CEA	Peptide	NCT00012246	Phase 2
Non Small Cell Lung	Dex2	Peptide	NCT01159288	Phase 2
Pancreatic	hTERT	Peptide	NCT00358566	Phase 3
Prostate	PSA	Peptide	NCT00109811	Phase 2
	PAP	Sipuleucel-T	NCT01477749	Phase 2
	PAP	Sipuleucel-T	NCT00005947	Phase 3
	PAP	Sipuleucel-T	NCT00715078	Phase 2
	PAP	Sipuleucel-T	NCT01338012	Phase 2
	PAP	Sipuleucel-T	NCT00065442	Phase 3
	PAP	Sipuleucel-T	NCT00901342	Phase 2
	PSA	Peptide	NCT00030602	Phase 2
	PAP	Sipuleucel-T	NCT01431391	Phase 2
Renal Cell	gp100; MART-1; tyrosinase	Peptide	NCT00019396	Phase 2
	Multiple IMA901	Peptide	NCT00523159	Phase 2
	Multiple IMA901	Peptide	NCT01265901	Phase 3

Table 3. Cancer vaccines in Phase 3, completed or terminated, based on peptide/protein strategies.

CANCER	Antigen	STRATEGY	NCT NUMBER	STATUS	Outcome
Breast	MUC1	Peptide	NCT00925548	Terminated	Following the clinical hold, EMD Serono has decided to permanently terminate the trial EMR 200038–010 (STRIDE) in the indication of breast cancer
Esophageal/Gastric	G17DT	Peptide	NCT00020787	Completed	Data not available
Melanoma	tyrosinase	Peptide	NCT01989572	Completed	Data not yet available
	MART-1; NA17-A; gp100; tyrosinase	Peptide	NCT00036816	Terminated	Low accrual
	gp100	Peptide	NCT00019682	Completed	In patients with advanced melanoma, the response rate was higher and progression-free survival longer with vaccine and interleukin-2 than with interleukin-2 alone.
Pancreatic	hTERT	Peptide	NCT00358566	Completed	Preliminary data showed no survival benefit in the GV1001 group compared to the gemcitabine group
Prostate	PAP	Sipuleucel-T	NCT00005947	Completed	Data for FDA registration
	PAP	Sipuleucel-T	NCT00065442	Completed	Data for FDA registration

pathogen- or host-derived antigenic proteins, which are processed and presented to naïve T lymphocytes at the lymphoid organs in the context of major histocompatibility (MHC) molecules.[135]

Several cancer immunotherapeutic strategies have been developed based on DCs (reviewed in[132]) stemming from the original works on generation of *ex vivo* DCs from mice, starting from bone marrow precursors,[136] and later on from humans, starting from CD34+ haematopoietic progenitors or from peripheral blood–derived monocytes.[137] *Ex vivo* generated DCs have been loaded with different sources of antigens mostly targeting melanoma, including whole tumor cells[138-141] and tumor-derived proteins or peptides.[142-144] Several clinical trials have been conducted along the years with DCs loaded with tumor-derived specific targeting melanoma,[145-147] renal cell carcinoma[148] and glioma,[149,150] resulting in contrasting clinical outcomes.

The Sipuleucel-T (Provenge^{TM}) is an "immune cell"-based cancer vaccine targeting prostate cancer consisting of autologous whole immune cell population incubated with PA2024 that contains prostatic acid phosphatase (PAP, a prostate antigen) fused to GMCSF.[97,96] In 2010 it was the first therapeutic cancer vaccine ever approved by the US FDA and its application is for the treatment of asymptomatic metastatic castrate-resistant prostate cancer (mCRPC).[151] However, no difference in time to progression is observed and a modest 4.1-month improvement in

Table 4. Cancer vaccines in clinical trials based on nucleic acids strategies.

CANCER	Antigen	STRATEGY	NCT NUMBER	PHASE
Acute Myeloid Leukemia	WT-1	RNA-pulsed DC	NCT01686334	Phase 2
	WT-1	RNA-pulsed DC	NCT00834002	Phase 1
Breast	Multiple antigens	DNA vaccine	NCT02157051	Phase 1
	CEA	RNA-pulsed DC	NCT00003432	Phase 1/2
Colorectal	CEA	RNA-pulsed DC	NCT00003433	Phase 1/2
Kidney	hPSMA	DNA	NCT00096629	Phase 1
Lymphoma	Idiotype	DNA	NCT01209871	Phase 1
Melanoma	Multiple	RNA	NCT00204516	Phase 1/2
	tyrosinase-related peptide 2 (TRP2)	RNA-pulsed DC	NCT01456104	Phase 1
	Neo-antigens	RNA	NCT01684241	Phase 1
	Neo-antigens	RNA	NCT02035956	Phase 1
	gp100 and tyrosinase	RNA-pulsed DC	NCT00940004	Phase 1/2
	gp100 and tyrosinase	RNA-pulsed DC	NCT00243529	Phase 1/2
	Multiple	RNA-pulsed DC	NCT01216436	Phase 1
	gp100 and tyrosinase	RNA-pulsed DC	NCT01530698	Phase 1/2
	Multiple	RNA-pulsed DC	NCT00672542	Phase 1
Multiple	CEA	RNA-pulsed DC	NCT00004604	Phase 1
	NY-ESO-1	DNA	NCT00199849	Phase 1
Non Small Cell Lung	Multiple	RNA	NCT00923312	Phase 1/2
	Multiple	RNA	NCT01915524	Phase 1
Prostate	Multiple	RNA	NCT00906243	Phase 1/2
	PSA	DNA	NCT00859729	Phase 1/2
	PSA	RNA-pulsed DC	NCT00004211	Phase 1/2

Table 5. Cancer vaccines in clinical trials based on viral vector strategies.

CANCER	ANTIGEN	STRATEGY	NCT NUMBER	PHASE
Bladder	PANVAC	Vaccinia/Fowlpox	NCT02015104	Phase 2
Brain/CNS	CEA	measles virus	NCT00390299	Phase 1
Breast	CEA & MUC-1	Vaccinia/Fowlpox	NCT00179309	Phase 2
	HER-2/Neu	Adenovirus	NCT00197522	Phase 1
Melanoma	gp100 antigen	Fowlpox	NCT00019175	Phase 1
	gp100 antigen	Fowlpox	NCT00019669	Phase 2
	tyrosinase	Fowlpox	NCT00019734	Phase 2
	tyrosinase	Fowlpox	NCT00054535	Phase 2
	multiple	ALVAC	NCT00613509	Phase 2
Multiple	MUC-1	MVA	NCT00004881	Phase 1
	EBNA1/LMP2	MVA	NCT01147991	Phase 1
	HER-2/Neu	Adenovirus	NCT01730118	Phase 1
	CEA	Fowlpox	NCT00217373	Phase 1
Nasopharyngeal	EBNA1/LMP2	MVA	NCT01256853	Phase 1
Non Small Cell Lung	MUC-1	MVA	NCT01383148	Phase 2b/3
Ovarian	NY-ESO-1	Fowlpox	NCT00112957	Phase 2
	NY-ESO-1	ALVAC	NCT00803569	Phase 1
	CEA	Measles virus	NCT00408590	Phase 1
	NY-ESO-1	ALVAC	NCT01982487	Phase 1/2
Pancreatic	CEA, MUC1, and TRICOM	Vaccinia/Fowlpox	NCT00088660	Phase 3
Prostate	5T4	Poxvirus	NCT01194960	Phase 2
	PSA	Fowlpox	NCT00005039	Phase 2
	PSA	Fowlpox	NCT00450463	Phase 2
	PSA	Fowlpox	NCT00045227	Phase 2
	PSA	Adenovirus	NCT00583024	Phase 2
	PSA	Fowlpox	NCT00020254	Phase 2
	PSA	Fowlpox	NCT00003871	Phase 2
	PSA	Vaccinia	NCT00001382	Phase 1
	PSA, TRICOM	Vaccinia/Fowlpox	NCT01322490	Phase 3

median survival in the active arm with respect to the placebo arm was observed (25.8 vs. Twenty-one.7 months).

Although the registration of Sipuleucel-T as therapeutic cancer vaccine represents a great advancement in the cancer immunotherapy field, the modest efficacy urges improvements and optimizations of the DC-based strategy. Increasing expression of activating molecules or, on the other side, reducing expression of inhibitory molecules would result in improved capacity of DCs in stimulating T cell activation and, ultimately, in anti-tumor efficacy. Over-expression of CD40L in human DCs results in increased elicitation of T cell response to tumor antigens, such as glycoprotein 100 (gp100) and Melan A.[152,153] Similarly, enhanced DC functions in stimulating antigen-specific Th1 and CTL responses can be achieved by modulation of other costimulatory molecules or proinflammatory factors.[154-159] Conversely, silencing of the ubiquitin-editing enzyme A20 or the scavenger receptor SRA/CD204 in human DCs facilitates the development of IFN-γ producing Th1 cells and antigen specific CD8+ T cells.[160-163] These findings suggest that the potency of current DC vaccines can be efficiently optimized resulting in improved clinical outcomes.

Additional strategies for antigen-specific vaccines

Alternative strategies to deliver antigen or antigen fragments *in vivo* is to utilize genetic vaccines or viral vectors (**Table 4 and 5**). These strategies, indeed, allow the delivery of multiple antigens with the activation of various arms of immunity (reviewed in[164,165]).

DNA vaccine platforms have shown promise in preclinical studies[166] which, however, do not hold when translated to non-human primates and humans[167,168] due to lack of efficacy. New constructs and methods of administration may enhance their efficacy. Indeed, Phase I/II trials for melanoma and other cancers are currently testing the efficacy of DNA vaccines injected directly into the lymph nodes, aiming at increasing antigen uptake by APCs and promote local inflammatory signals.[169,170] However, the *in vivo* nucleic acid electro-gene-transfer (EGT) appears to be the most promising strategy to enhance immunogenicity of nucleic acid immunizations for cancer vaccine protocols[171] and a list of the ongoing cancer vaccine clinical trials with use of electro-gene-transfer is reviewed in.[172]

Similar to DNA vaccines and viral vectors, RNA vaccines may induce both CD4+ and CD8+ T cell responses and candidates targeting cancer antigens have been evaluated.[173–175]

mRNA vaccine candidates have been tested in human clinical trials using either whole tumor cell transcriptome[176] to target metastatic melanoma, or specific TAAs to target metastatic melanoma[177] and renal cell carcinoma,[178] eliciting tumor antigen-specific antibody and T cell responses. More recently, trials targeting prostate and non-small cell lung cancer have shown mRNA vaccines to be safe, well tolerated and immunogenic.[179]

The first and most extensively evaluated viral-based vectors in cancer vaccine trials are from the poxviridae family, such as vaccinia, modified vaccinia strain Ankara (MVA), and the avipoxviruses (fowlpox and canarypox; ALVAC).[180,181] PROSTVAC is a

cancer vaccine to prostate cancer based on a replication-competent vaccinia prime and a replication-incompetent fowlpox boost. Each vector contains transgenes for PSA and 3 costimulatory molecules (CD80, CD54 and CD58), designated TRICOM.[182] In 2 independent phase II trials, PROSTVAC improved median overall survival relative to the control vector[183,184] and a phase III trial is currently ongoing (NCT01322490).

The MVA vector-based cancer vaccine TG4010 targeting the MUC1 antigen has been tested in a phase II trial for renal cell carcinoma combined with interferon-α2a and IL-2, resulting in improved overall survival.[185] A separate phase II trial of TG4010 combined with first-line chemotherapy (cisplatin plus gemcitabine) in advanced NSCLC demonstrated a significant 6 months increase in median survival.[186] A confirmatory phase IIb/III trial of TG4010 for treatment of advanced stage (IV) NSCLC is ongoing (NCT01383148).

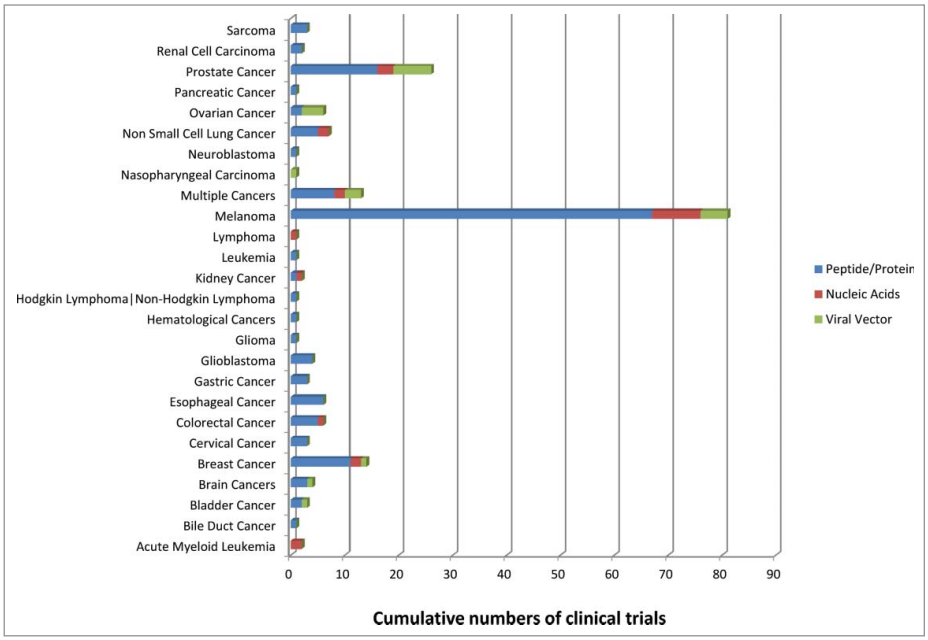

Figure 1. Cumulative numbers of cancer vaccine clinical trials for each cancer and each vaccination strategy.

A phase III clinical trial has been conducted and terminated to evaluate the efficacy of PANVAC-VF, a vaccine composed of recombinant vaccinia virus and fowlpox virus expressing CEA, MUC1, and TRICOM, in patients with advanced pancreatic cancer (NCT00088660). Vaccinated patients failed to show an advantage in overall survival over standard palliative chemotherapy.[187]

Adenovirus vectors expressing various TAAs (PSA, HER-2/Neu) are currently being tested for their immunological and clinical efficacy (NCT00583024, NCT00197522). Moreover, an adenovirus expressing the extracellular and transmembrane domains of HER2 is currently evaluated in patients with any HER2-expressing tumor, aiming at inducing neutralizing antibodies against HER2, not T cells (NCT01730118).

Conclusions and Future Directions

Several cancer vaccines clinical trials have been conducted in the last years based on the different type of antigens described in the present review (peptide vs. genetis vs. viral vectors). The vast majority of such clinical trials have been based on peptides mostly targeting melanoma (**Fig. 1**). The prevalence of peptide-based clinical trials is observed also in the different phases of clinical trials (**Fig. 2**). To date, only few clinical trials have reached the efficacy Phase III evaluation, based only on peptides and viral vectors. Evaluation of cancer vaccines on an increased number of target cancers using diverse vaccine strategies would definitely be highly beneficial to improve the knowledge in the field and, ultimately, clinical outcome in cancer patients.

Indeed, the first therapeutic cancer vaccine approved by FDA for the treatment of asymptomatic metastatic castrate-resistant prostate cancer (Sipuleucel-T (Provenge™), represents a

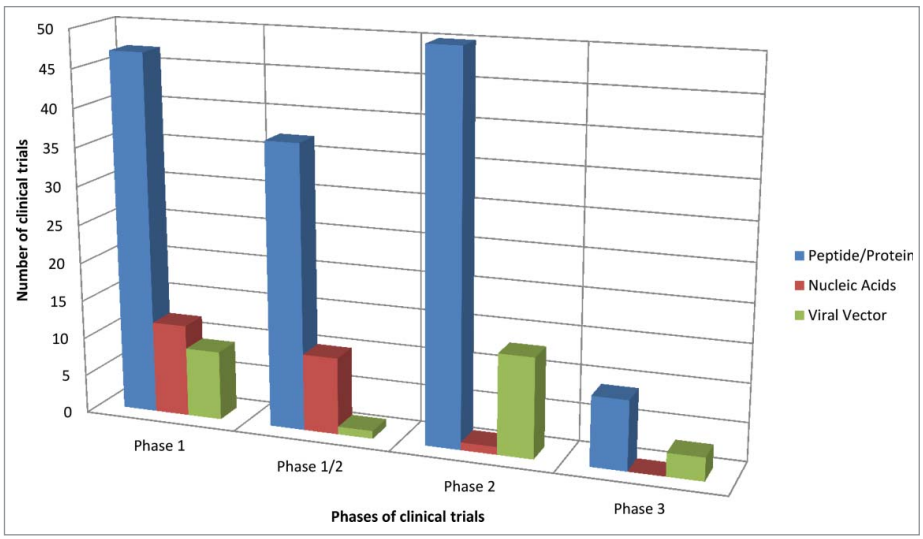

Figure 2. Number of cancer vaccine clinical trials in each experimental phase for each vaccination strategy.

landmark. However, Sipuleucel-T shows a modest increase in overall survival and other large scale clinical trials do not prove yet to be as efficacious as needed for complete tumor regression.

Several reasons account for these disappointing results. Identification of the appropriate target antigens, represents one the most relevant aspects and currently available high – throughput strategies make this goal accomplishable.

Along this path, identification of peptides naturally processed and presented by HLA molecules (***HLA ligandome***) on tumor cells as well as the personalized immunotherapy, to identify target tumor-associated antigens specific for each individual cancer patient, is further raising the bar in the quest of eliciting tumor specific immunity.

Efficacy in clinical application of cancer vaccine approaches based on cocktails of specific epitopes identified with high – throughput technologies is very promising and is currently being further evaluated in a broader range of tumors.

In general, besides target antigen identification, chances of success may increase only if a multi-faceted strategy is undertaken, including 1) addressing the tolerogenic environment and tumor suppressive mechanisms by combinatorial immunotherapy; 2) selecting optimal antigen presentation and delivery system; 3) adding a potent immune modulator able to increase the immunogenicity of the vaccine and to specifically elicit the more appropriate arm of the immune response (i.e. Th1 vs. Th2); and 4) employing multiparametric analyses to identify prediction markers of immunogenicity for selection of best responding vaccinees.

The combination of all such approaches will represent a great advancement in cancer vaccinology, enabling the development of vaccines with enhanced therapeutic efficacy to hopefully improve the quality of life of cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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