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Cancer immunotherapy: moving forward with peptide T cell vaccines

Takumi Kumai[(1),(2),(3),*], **Aaron Fan**[(1),*], **Yasuaki Harabuchi**[(2)], and **Esteban Celis**[(1)]

[(1)]Cancer Immunology, Inflammation and Tolerance Program, Georgia Cancer Center, Augusta University Augusta, GA

[(2)]Department of Otolaryngology-Head & Neck Surgery, Asahikawa Medical University

[(3)]Department of Innovative Head & Neck Cancer Research and Treatment (IHNCRT), Asahikawa Medical University

Abstract

Recent advances in cancer immunology, such as the discovery of immune checkpoint inhibitors, have validated immune cells as potential key players for effective cancer treatment. The efficacy of these therapies seems to be codependent on a tumor-reactive T lymphocyte response. For many years, numerous attempts and strategies in developing vaccines to generate tumor-reactive T cells have yielded poor results in the clinic due to suboptimal immunogenicity and the inability to overcome an immunosuppressive tumor microenvironment. In this review, we summarize past and current advances in T cell vaccines and describe our experience in developing optimized methods for antigen/adjuvant selection and vaccine administration in order to induce powerful anti-tumor responses.

1. Introduction

Undoubtedly, vaccines are effective in preventing infections by recruiting various components of the immune system against numerous pathogens. Since the immune system has the ability to recognize transformed malignant cells and limit tumor growth, immunotherapy has now become an effective way to treat cancer. Amongst various components of the immune system T cells and in particular CD8 cytotoxic T lymphocytes (CTLs) are the most effective elements in recognizing alterations occurring in transformed cells. The antigens recognized by T cells correspond to peptides that associate MHC molecules. Such peptides result from processed proteins from the infectious microorganisms or derived from abnormally expressed gene products in malignant cells. Tumor-reactive T

[*]These authors contributed equally to this manuscript.

Conflicts of interest

Esteban Celis has filed patent applications based on the use of synthetic peptides and poly-IC combinatorial vaccines. The rights of the patent applications have been transferred to the Moffitt Cancer Center (Tampa, FL). Other authors declare no conflict of interest.

cells are frequently present in cancer patients in the form of tumor-infiltrating lymphocytes (TILs), which normally do not control the disease [1]. However, *ex vivo* TIL expansion and reintroduction into the patients has demonstrated remarkable therapeutic effects in some patients [2]. Unfortunately TIL therapy is technically challenging, expensive and not all cancers contain TILs. Thus, there is a critical need for other means to generate tumor reactive T cells with a simpler and more cost effective strategy such as vaccination.

Checkpoint pathways regulate T cells by blocking their function, presumably to prevent pathological autoimmune responses [3]. Antibodies that inhibit two of these immune checkpoint blockades, CTLA4 and PD-1, have shown notable anti-tumor effects [4,5]. However, the proportion of patients that respond favorably to checkpoint blockade inhibitors (CBIs) is low and is confined to particular types of cancer. Because CBIs require the presence of an existing pool of tumor-reactive T lymphocytes, many believe that patients not responding to CBI lack these T cells. Thus, the expectation is that T cell inducing vaccines should increase the effectiveness and expand the applicability of CBIs.

2. Types of T cell vaccines

Various strategies have been used to develop vaccines to generate tumor-reactive T cells (Table 1). This work developed from early pioneering observations in mice where killed tumor cells vaccines prevented the growth of subsequent challenges with live tumor cells [6,7]. Nevertheless these vaccines were less effective when administered into animals bearing established tumors. Vaccines consisting of tumors expressing immune-stimulating cytokines improved their anti-tumor effects [8], but unfortunately the clinical results were not outstanding [9–12]. Thus, major efforts are devoted to designing more effective vaccines by utilizing defined tumor antigens (TAgs).

3. Antigen selection

The identification of proteins that function as TAgs for T cells and their corresponding peptide epitopes facilitated developing more refined T cell vaccines. Practically, TAgs for T cells are grouped into 4 types (Table 2): A) Products of oncogenic viruses; B) Developmental or germ cell products; 3) Tissue-specific differentiation antigens; 4) Products of genetic alterations derived from malignant transformation.

Tumor whole exome sequencing has allowed identifying mutations that potentially represent tumor-specific T cell epitopes [13]. Yet, such mutations must occur in protein-coding regions and be contained within peptides binding to MHC molecules. There are numerous algorithms used to predict whether a peptide binds to a specific MHC allele [14,15] and quantitative peptide/MHC-binding assays can be used to demonstrate the formation of peptide/MHC complexes [16]. However, whether a mutated MHC binding peptide is effective in generating tumor-reactive T cells depends on whether the mutated epitopes are generated by the tumor and expressed as surface peptide/MHC complexes. This fundamental requirement is challenging to demonstrate since it requires proof of the mutated peptide presence bound to the tumor MHC molecule, for example by mass spectrometry sequencing of peptides eluted from purified MHC proteins [17]. Alternatively, attesting tumor cell

recognition by T cells specific for mutated peptide would demonstrate this prerequisite. Because these approaches are technically challenging, many choose to select mutated peptides solely on the basis on MHC binding predictions to produce peptide cocktail vaccines, not taking into consideration the possibility that peptides not corresponding to true tumor T cell epitopes could generate irrelevant T cell responses that may diminish the effectiveness of the vaccine.

Although there are multiple *pros* and *cons* related to the use of TAgs from each group (Table 2), there is still no clear evidence that antigens from one of these types will be more effective than the others. The most significant issue for selecting a TAg is related to potential immune tolerance that could affect the quality of the T cell response (capacity to recognize the tumor cell). Nevertheless, there are examples of successfully eliciting anti-tumor T cell responses to “self-antigens”, when immune tolerance was predicted [18–21].

4. Immunization strategy

Whatever TAg type is selected, diverse vaccination approaches have been explored, such as recombinant proteins, recombinant viruses, DNA vaccines and synthetic peptides. These vaccines are directly administered *in vivo* with the expectation that professional antigen-presenting cells (pAPCs) such as dendritic cells (DCs) will capture vaccine components and stimulate T cell responses. In other cases the vaccine components are loaded *ex vivo* onto DCs to produce cell-based vaccines. The goal is that the DCs presenting the peptide/MHC complexes will stimulate T cells via their T cell receptor (TCR), inducing them to proliferate and become effector cells. However, it is evident that effective T cell activation leading to expansion, survival and effector function requires more than simple TCR stimulation (Signal 1). DCs need appropriate activation to provide immune costimulatory signals that promote T cell survival and proliferation after TCR stimulation. Costimulation occurs either in the form of cell surface receptor-ligand interactions between T cells and DCs (Signal 2), or via cytokines (Signal 3). Signal 1 without Signals 2 and 3 fails to generate effective T cell responses and leads to T cell dysfunction [22,23]. During an immune response to an infectious agent, DC activation results from stimulation of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), and RIG-I-like receptors (RLR) by microbial components [24]. Helper CD4 T lymphocytes (HTLs) further promote DC activation through CD40L/CD40 [25]. Consequently, effective CTL vaccines must contain immune adjuvants that stimulate PRRs, and should stimulate HTLs. Agonistic antibodies to CD40 have been used to enhance the effectiveness of CTL-inducing vaccines that may not trigger HTL responses [20,25,26].

We believe that examining how the immune system responds to an acute infection could facilitate developing effective cancer vaccines that elicit large and lasting CTL responses. During the course of an immune response to infectious pathogen, the activated CTLs will encounter antigen again on infected non-professional APCs cells (e.g., epithelial cells). Under these circumstances, production of type-I interferon (IFN-I) by the infected cells will function as Signal 3 allowing the CTLs to survive and continue to expand until the infection is resolved [27,28]. These basic concepts could be applied for developing cancer vaccines.

5. Optimization of peptide vaccines

Many favor synthetic peptides as the source of TAg for developing vaccines because they are easy to manufacture and characterize in a cost-effective manner for the clinic. Unfortunately, numerous clinical studies using peptide vaccines have resulted in suboptimal therapeutic benefit in most patients [29–31]. We attribute this failure to two major causes: 1) suboptimal immunogenicity, and 2) the presence of an immunosuppressive tumor microenvironment.

Historically vaccines were developed towards eliciting antibodies to prevent infections. Thus, vaccine optimization and evaluation relied in measuring antibody titers, leading to the selection of immune adjuvants and modes of administration, inducing high antibody titers that correlated with protection. Regrettably, cancer vaccine development has followed the same approach to induce CTL responses using the same adjuvants and modes of administration. Proteins and peptides corresponding TAgs are administered with adjuvants such as alum or emulsified in incomplete Freund's adjuvant and injected subcutaneously to generate CTLs. Moreover, evaluating CTL responses is far more complex than measuring antibody titters, and some approaches lead to erroneous assumptions of the vaccine's immunogenicity. Ideally, one should quantify numbers of antigen-specific tumor-reactive CTLs before and after vaccination. However, CTL tumor-reactivity assays are difficult to perform since these necessitate a substantial amount of blood and require tumor cells as target cells. Thus, many studies use assays measuring T cell responses (cytokine release) to TAg used in the vaccine (peptide, protein) presented by conventional APCs, or assess the presence of antigen-specific T cells by flow cytometry using peptide/MHC tetramer staining. These methods do not evaluate the ability of the vaccine-induced antigen-reactive T cells to recognize tumor cells. Some conclude that a vaccine is immunogenic solely in the basis of "statistically significant" differences in numbers of antigen-reactive T cells between pre- and post-vaccination samples without taking into account whether these differences are truly substantial to hold any biological relevance. What constitutes a biological relevant CTL response leading to a meaningful therapeutic benefit? Many factors will influence the antitumor effectiveness of the T cell response. In addition to the capacity of the CTLs to recognize the tumor cells (quality), the ability of the T cells to traffic and infiltrate the tumor and to withstand the inhibitory microenvironment will dictate the necessary numbers of CTLs required controlling tumor growth. Nevertheless, one can predict that the efficacy of the vaccine's antitumor effect will depend on the magnitude and quality of the CTL response.

Based on the work of other [26], we developed a synthetic peptide vaccination strategy (TriVax) that induced rapid and vast CTL responses in mice, where in many instances 50–80% of all CD8 T cells were specific for the peptide immunogen and recognized tumor cells [20,32]. In addition to peptide, TriVax contains a PRR agonist (poly-IC) and a DC-activating antibody (anti-CD40 mAb). Later we described a simpler vaccine (BiVax), using amphiphilic peptides that self-assembled into nanostructures, which were administered with poly-IC without the anti-CD40 mAb [33]. Two sequential immunizations (prime/boost) were required for generating large CTL responses. These vaccines had to be injected systemically (intravenously or intramuscularly), which disseminates the antigen and adjuvant throughout the lymphoid organs, optimizing the recruitment of naive CTLs. Amongst several PRRs

tested as adjuvants, poly-IC was unique in promoting vast CTL expansions [20,33]. The effectiveness of poly-IC as an adjuvant relies in its capacity to stimulate TLR3, shown to be important during priming, and its ability to stimulate MDA5, a cytoplasmic RLR responsible for generating IFN-I [34], which was critical for the boost CTL expansions. TriVax and BiVax were effective against established tumors in mice. In a model of human papilloma virus (HPV)-induced tumors, therapeutic immunization using CTL epitope HPV16-E7$_{49–57}$ resulted in eradication of tumors [32]. With the less immunogenic B16-F10 murine melanoma vaccines using several melanosomal derived CTL epitopes (Trp2$_{180–188}$, Trp1$_{455–463}$ and gp100$_{25–33}$) significantly reduced growth of established tumors and tumors were rejected when vaccines were combined with PD-1 blockade [20,33].

Some propose the use of long synthetic peptide vaccines because this would force antigen presentation by DCs [35]. Although this strategy makes sense, in our view the use of potent adjuvants and appropriate routes of administration are more critical than simple peptide length. We believe that physical properties of peptides resulting from their amino acid sequence and composition will determine the immunogenicity of a vaccine. For example the minimal CTL epitope HPV16-E7$_{49–57}$ (RAHYNIVTF) was significantly more immunogenic (10 to 100-fold) with BiVax as compared to 2 long peptides, HPV16-E7$_{45–57}$ and HPV16-E7$_{43–77}$ [33]. The minimal epitope is amphiphilic, since one end is hydrophilic (RAHYN) while the other is hydrophobic (IVTF), and when this peptide is resuspended in an aqueous solvent it self-associates into virus resembling nanoparticles. On the other hand, the elongated peptides HPV16-E7$_{45–57}$ and HPV16-E7$_{43–77}$ are not amphiphilic and are water soluble, which could result in rapid clearance and lower immunogenicity. Following this rationale, the immunogenicity of several non-amphiphilic minimal CTL epitopes was improved by elongating them to form amphiphiles [33].

In addition to helping CTL responses, CD4 HTLs display direct antitumor effector function. In many instances HTLs are effective in killing MHC class II (MHC-II) expressing tumor cells [36], but in other cases, HTLs can eradicate MHC-II negative tumor cells [37]. Our group has been involved in identifying MHC-II binding peptides from numerous human tumor antigens capable of functioning as HTL epitopes [19]. Many of these peptides function as promiscuous epitopes (can be presented by several MHC-II alleles), potentially allowing their use as vaccines in broad patient populations. Following the model used with CTL vaccines, we reported the use of TriVax for eliciting substantive HTL responses in mice [21]. As with CTLs, the induction of HTLs by TriVax required a systemic prime-boost peptide administration and relied on IFN-I. HTL responses by TriVax were more effective with a TLR7 agonist instead of poly-IC and were enhanced by an OX40 agonist antibody. With a peptide derived from Trp1 (Trp1$_{113–127}$), TriVax was able to overcome immune tolerance and induced HTLs capable of recognizing and killing MHC-II-expressing (interferon-gamma treated) B16 melanoma cells. TriVax immunization with the Trp1 HTL epitope into mice with established B16 tumors resulted in significant reductions in the rate of tumor growth [21].

6. Future challenges for peptide vaccines

Overall, our results demonstrate that peptide vaccines can be an effective way of inducing strong CTL and HTL anti-tumor responses in mice when administered with the appropriate adjuvants and immunization routes. However, it remains to be determined whether TriVax, BiVax or similar vaccination strategies will be effective in patients. There are several necessary prerequisites in order to take TriVax or BiVax into the clinic. Although synthetic peptides are relatively easy and cost effective to produce under good manufacturing practices (GMP), amphiphilic peptides, especially those containing palmitic acid chains can pose a challenge to purify using conventional methods. While currently there are no FDA-approved humanized anti-human CD40 agonistic antibodies, there are several under development, which could be used for TriVax. A GMP grade formulation of poly-IC (Hiltonol™ from Oncovir, Inc.) that is stabilized with poly-lysine and carboxymethylcellulose (poly-ICLC) has been extensively used in the clinic as an experimental drug to induce IFN-I to treat multiple sclerosis and glioblastoma and has been recently used as an immune adjuvant. We have experimental evidence in mice that poly-ICLC is a more potent adjuvant for T cell responses as compared to poly-IC (E. Celis, unpublished observations). Lastly, there is some reticence for administering vaccines intravenously due to safety concerns. Nevertheless, both TriVax and BiVax are effective in mice when injected via an intramuscular route, which also disseminates the peptide, poly-IC and antibody systemically to reach most antigen-specific T cell precursors.

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Abbreviations

APC	antigen-presenting cell
CBI	checkpoint inhibitor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
GMP	good manufacturing practices
HTL	helper T lymphocyte
HPV	human papilloma virus
IFN-I	type-I interferon
pAPC	professional antigen presenting cell
PRR	pathogen recognition receptor
RIG-I	retinoic acid inducible gene-I

RLR	RIG-I-like receptor
TAg	tumor antigen
TCR	T cell receptor
TIL	tumor-infiltrating lymphocyte
TLR	toll-like receptor

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*of special interest

**of outstanding interest

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Highlights

1. Cancer vaccines have not generated effective anti-tumor responses due to suboptimal immunogenicity.
2. Peptide vaccines with appropriate adjuvants are the most promising strategy to induce tumor-reactive T cells and treat cancer.
3. The combination of optimized peptides, strong adjuvants and costimulatory molecule stimulation together with systemic immunizations results in robust T cell responses.
4. Therapeutic peptide-based vaccines can reduce the tumor growth by generating tumor-specific CD8 CTLs or CD4 HTLs.
5. Blocking the immunosuppressive tumor microenvironment potentiates the effectiveness of cancer vaccines.

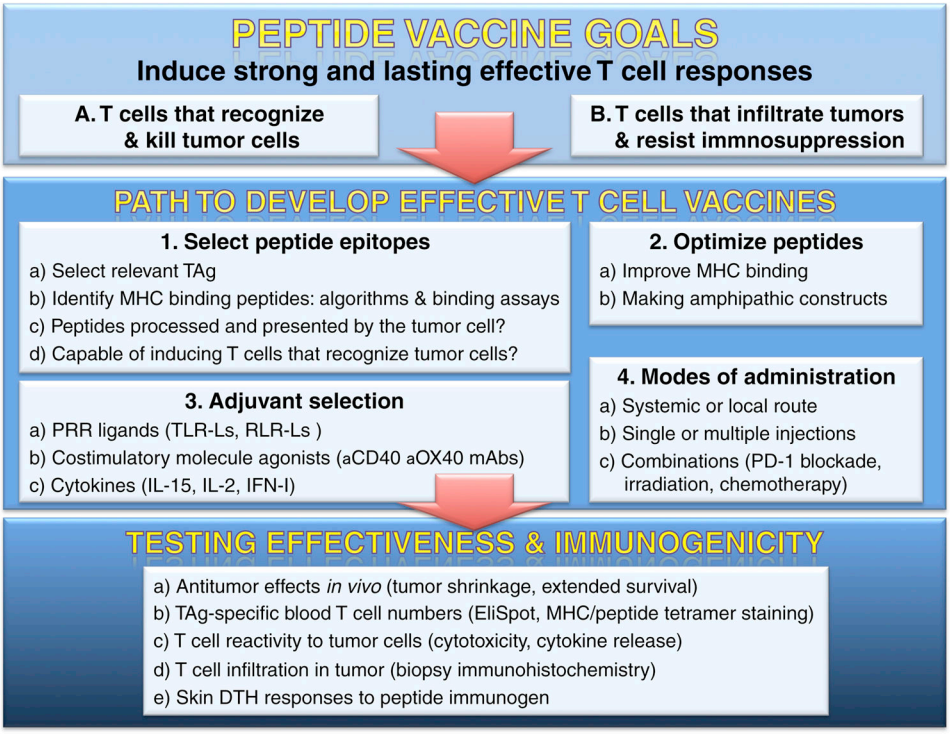

Figure 1. Rational path to designing and testing effective T cell epitope-based vaccines for cancer

The goals of tumor vaccines are to induce strong and lasting antitumor T cells that can recognize and kill tumor cells (A), and overcome immunosuppression in the tumor microenvironment (B). The 4 steps to develop the vaccines: 1) Epitope selection is critical to develop effective T cell epitope-based vaccines. TAg potential epitopes that bind to an MHC molecule are predicted using computer-based algorithms and can be validated with binding assays. The presence of the peptide epitope on tumor cells can be assessed with mass spec sequencing of MHC eluted peptides. Epitope immunogenicity is established by *in vitro* T cell stimulation assays or *in vivo* by vaccinating HLA transgenic mice. 2) To enhance immunogenicity the amino acid sequence of epitope can be modified to increase MHC binding or enhance amphiphilicity. 3) The selection of appropriate adjuvants, costimulatory agonists and cytokines will determine the magnitude and duration of the T cell responses. 4) The mode of vaccine administration (injection route, boosters) and the possibility combining the vaccine with adjunct treatments are factors to consider for achieving effective antitumor responses. The immunogenicity and effectiveness of vaccines should be monitored by measuring realistic antitumor effects in vivo (a), changes in TAg-specific T cells pre- and post-vaccination (b), assessing T cell reactivity to tumor cells (c), infiltration of T cells in tumors (d), and evaluating skin delayed type hypersensitivity (DTH) responses in the vaccinees.

Table 1

Development of vaccines to generate tumor-reactive T cells

Strategy	General Overview	Reference
Cell-based vaccines		
Tumor cells/lysate	Killed autologous tumor, cancer cell lines or cell lysates	7, 38
Genetically modified tumor cells	Tumor cell lines bioengineered to express cytokines (e.g. GM-CSF, IL- 4)	8–12, 39
Dendritic cells	DCs are isolated or prepared *ex vivo* and loaded with TAg and then injected to patients	40
Microorganism-based vaccines		
Recombinant viruses	Attenuated viruses that encode TAg	41–43
Recombinant bacteria	Attenuated, TAg-expressing bacteria (Listeria, Salmonella)	44–45
Recombinant yeast	Recombinant yeast particles expressing TAg on their surface enhance and promote presentation of TAg by APCs while avoiding risks associated with live pathogen vaccine models	46
Subunit vaccines		
Peptides, proteins	Short, or long TAg-derived; synthetic peptides including helper epitopes; carbohydrate- mimetic peptides	20, 21, 26, 30–33, 35, 47
DNA or RNA	DNA plasmids or RNA encoding TAg or identified T cell epitopes injected to transduce host cells to express TAg	48, 49
Heat shock proteins	HSP-TAg complexes isolated from patient tumor extracts to target TAg to APCs	50

Table 2

Types of tumor antigens for T cells

TAg Type	Pros	Cons	Examples
A. Products of oncogenic viruses	High immunogenicity (no tolerance)	The target tumors are limited	EBV, HBV, HPV
B. Developmental or germ cell products	Lower risk of recognizing self-antigens in normal tissue (low tolerance) TAgs can be pharmacologically induced in tumor cells	Possibility of adverse effects to reproductive organs???	Tumor-testis antigens (MAGE-A3, NY- ESO1)
C. Tissue-specific differentiation antigens	High specificity, lower risk of off-target effects	The target tumors are limited, possibility of adverse effects to normal tissues Low immunogenicity (tolerance)	Melanosomal proteins (gp100, Trp1, Melan- A/Mart-1), prostatic proteins (PSA, PAP)
D. Products of genetic alterations derived from malignant transformation	High immunogenicity	The identification of neoepitopes in each individual (i.e. expensive and complicated)	a) Overexpressed gene products (HER2/*NEU,* CEA b) Mutation-derived epitopes (P53, Ras, EGFRvIII, BCR-ABL, point mutations/neoepitopes)