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REVIEW

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Monitoring tumor antigen specific T-cell responses in cancer patients and phase I clinical trials of peptide-based vaccination

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Abstract Numerous phase I and II clinical trials testing the safety and immunogenicity of various peptide vaccine formulations based on CTL-defined tumor antigens in cancer patients have been reported during the last 7 years. While specific T-cell responses can be detected in a variable fraction of immunized patients, an even smaller but significant fraction of these patients have objective tumor responses. Efficient therapeutic vaccination should aim at boosting naturally occurring antitumor T- and B-cell responses and at sustaining a large number of tumor antigen specific and fully functional effector T cells at tumor sites. Recent progress in our ability to quantitatively and qualitatively monitor tumor antigen specific CD8 T-cell responses will greatly help in making rapid progress in this field.

Keywords Cancer vaccines · Tumor immunology · Immune monitoring · Tetramers

The concept of defined therapeutic cancer vaccines

The identification of tumor-associated antigens recognized by cytolytic T lymphocytes (CTLs) at the molecular level formed the basis for the design of well-defined therapeutic cancer vaccines. Indeed, a growing number of phase I and II clinical trials are being performed and reported during the last decade. Some of the most often used approaches to experimental vaccination in human cancer patients include the use of synthetic peptides administered either free in aqueous solution or together with adjuvants, highly purified recombinant proteins, recombinant viral or bacterial vectors and autologous dendritic cells either loaded with peptides, proteins, or infected with recombinant vectors or even fused with tumor cells. In contrast to all the other vaccine delivery systems for CTL-defined antigens, peptides are by far the simplest compounds that lend themselves to rigorous chemical definition and pharmacological formulation. However, at the same time, peptides by themselves are rather weak immunogens. Hence the need to add immune adjuvants to boost their immunogenicity. In this minireview we will focus on the recent reports on clinical trials of peptide vaccination (Table 1). The majority of these trials share two common outcomes. On the one hand, specific immune responses were induced in a variable proportion of vaccinated patients, ranging from about 50% to 80% in some reports. On the other hand, a smaller proportion of vaccinated patients had measurable clinical benefit. In general the rate of clinical responses is not superior to 10–20%.

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The hurdles for cancer vaccines based on CTL-defined tumor antigens

Although therapeutic vaccination in cancer patients does lead to the induction of specific immunity in a significant but variable proportion of patients, consistent immunization leading to induction of CTL responses in *all* vaccinated patients remains to be achieved. More importantly, the low but significant level of clinical efficacy in the trials reported thus far suggests a lack of correlation between specific immunity measured in blood cells and antitumor effect. Thus, the

Table 1 Clinical trials of tumor antigenic peptide-based vaccination in mostly, but not exclusively, metastatic melanoma

Peptide	HLA	Adjuvant	CTL	Tumor response	Reference
MAGE-A3$_{168–176}$ Same peptide or recombinant minigene virus	A1	None	Not detected4/9 regressors and 1/14 progressors	7/25	[42] [13]
Melan-A$_{26–35}$ Tyrosinase$_{1–9}$ Tyrosinase$_{368–376}$ gp100$_{280–288}$gp100$_{457–466}$ Influenza matrix$_{58–66}$	A2	None or GM-CSF	3/62/60/60/6	3/3	[43]
Melan-A$_{27–35}$	A2	IFA	12/1815/18 by IFN-γ release	0/23	[24]
gp100$_{209–217}$	A2	IFA	2/8	1/19	[25]
gp100$_{209–217(T210\ M)}$	A2	IFA	10/11	0/11	[25]
gp100$_{209–217(T210\ M)}$	A2	IL-2 + IFA	3/19	8/195/12	[25]
MUC-1 peptide conjugated to KLH	A1, A2, A11	Detox-B	7/11	Not reported	[44]
gp100$_{209–217(T210\ M)}$ Tyrosinase$_{368–376\ (370D)}$	A2	IFA ± IL-12	33/38 by IFN-γ ELISA 37/42 by tetramer assay	Inconclusive	[45]
HPV16 E7$_{11–20,}$ E7$_{86–93}$ Pan-DR binding peptide	A2	IFA	ND	2/19	[46]
Bcr-abl breakpoint peptides (5 peptides)	Various	QS-21	Undetectable CTL proliferative responses	Not reported	[47]
Tyrosinase peptides	A1, A2,A3	GM-CSF	4/7	1/5	[48]
HPV E7$_{12–20}$	A2	IFA	10/16	3/18	[49]
HER-2$_{369–384,\ 688–703,\ 971–984}$	A2	GM-CSF	10/15, 5/15, 12/15	Not reported	[50]
gp100$_{209–217(T210\ M)}$ + HPV16 E7$_{11–20}$	A2	IFA	28/29	Not applicable	[14]
gp100$_{209–217(T210\ M)}$ and Melan-A$_{26–35(A27L)}$ ± gp100$_{44–59}$	A2DRB1*0401	IFA	18/19 (gp100) and 0/18 (Melan-A de novo)7/10 (gp100)0/10 (new anti-Melan-A)	0/221/19	[51]
MAGE-A12$_{170–178}$	Cw*0702	IFA	Not detected	1/9	[52]
gp100$_{209–217(T210\ M)}$ Melan-A$_{26–35\ (A27L)}$ Tyrosinase$_{368–376\ (370D)}$	A2	IFA + SD-9427 (Progenipoietin)	7/10 by IFN-γ to at least one or more peptides11/12 by tetramer assay (after in vitro stimulation)	Tumor free at study entry 4/15 relapsed with a median follow-up of 20 months	[53]
One to four tumor-associated peptides	A2 or A24	IFA	7/14 by IFN-γ ELISA; 8/12 by serology (IgG)	No objective tumor responses reported	[54]
Melan-A$_{27–35}$	A2	Peptide pulsed autologous PBMCs + rhIL-12	12/18 15/18 by IFN-γ release	7/20	[55]
gp100$_{209–217(T210\ M)}$ + gp100$_{280–288(A288\ V)}$	A2	IFA and systemic anti-CTLA-4	11/116/11	3/14 + severe autoimmunity	[56]

hurdles ahead for the development of effective therapeutic cancer vaccines are considerable and include (1) identification of ''universal'' tumor rejection antigens, (2) optimization of vaccination procedures, (3) quantitative monitoring of Ag-specific CTL responses, (4) identification of the type of protective CTL response(s), and (5) understanding tumor escape pathways.

The ideal tumor antigen for vaccination

Such antigens should display selective expression by all tumor cells and not by normal tissues; their expression should be essential for tumor cell survival; they should be targeted by both CD8 and CD4 T cells; and their recognition should be restricted by the most frequently expressed HLA alleles. Moreover, the corresponding peptides should be efficiently processed by the tumor cells and achieve a high density as MHC-peptide complexes at the surface of the tumor cells, and the level of immunological tolerance to these antigens should be minimal. It is clear that, for the time being, there are no tumor antigens that fulfill these stringent conditions. Thus, there is room for tumor antigen identification. In the meantime, the relatively large number of tumor antigens allows us to

choose those that come close to meeting the criteria listed above.

Therapeutic cancer vaccine optimization

The main goal today for the optimization of therapeutic cancer vaccines should be to achieve a 100% success rate in the induction of specific T-cell mediated immunity. In this regard, the use of molecularly defined tumor antigens for vaccination offers the opportunity to monitor the optimization process. The variables include the choice of the delivery system—naked DNA, recombinant vectors, short peptides, long peptides, recombinant protein or autologous dendritic cells loaded with an appropriate form of antigen—dose, route of administration, frequency of vaccination, and immunological adjuvants. Although, some of these parameters could be addressed in HLA transgenic mouse models, the most direct way for vaccine optimization is the implementation of well-planned, small phase I/II clinical trials with defined immune response endpoints.

Monitoring tumor antigen-specific CTL responses in vaccination

Unlike most prophylactic vaccines against infectious pathogens that aim at the induction of a predefined level of neutralizing antibodies, therapeutic vaccines based on CTL-defined antigens aim at inducing a specific CTL response. Simple and reliable assays to accurately measure antibody levels have been available to clinical immunologists for decades. They are routinely used in the clinics as well as in large scale clinical trials of vaccination [1, 2, 3]. In sharp contrast, assays to measure directly the numbers as well as the quality of antigen-specific T cells only became available 7 years ago. The introduction of soluble fluorescent class I MHC–peptide complexes, commonly known as tetramers or multimers, allowed us to enumerate CTLs and determine their relative frequencies directly ex vivo using flow cytometry [4]. However, they do not provide information on the differentiation stage of antigen-specific T cells. In this regard, the parallel development of single cell cytokine release assays has allowed us to simultaneously assess the effector capabilities of T cells of defined specificity. ELISpot assays for cytokine-producing T cells enable the rapid and relatively sensitive enumeration of T cells but do not provide direct information on the fraction of antigen-specific T cells with cytokine production capability. Flow cytometry–based cytokine-release assays offer the possibility to combine detection of single cytokine-secreting cells, with identification of those T cells binding defined class I MHC–peptide multimers. Two such assays exist which have been shown to fulfill this purpose: intracellular cytokine staining [5] and cytokine release assays [6]. The latter permits the visualization of intact cytokine-secreting antigen-specific T cells which can be isolated by flow cytometry cell sorting for analysis or for adoptive transfer immunotherapy [7]. In addition to cytokine production, T cells can also be characterized in terms of their cell surface phenotype. The expression of surface antigens such as CD45RA, CD45RO, CD27, CD28, and CCR7 allows us to establish correlations with their functional state of differentiation [8, 9, 10]. The expression of molecules associated with T-cell effector function can also be quantitated by intracellular staining such as perforin and granzyme B. Although, there are no single cell assays for the detection of multimer$^+$ T cells with lytic function, some recent methods designed to detect target cell apoptosis by flow cytometry may be useful to develop them [11].

Despite the advances in recent years in accurate monitoring of antigen-specific CD8 T-cell responses, progress is still needed. The assays remain labor intensive and are difficult to standardize. The high number of cells required limits the number of assays that can be performed. Moreover, the circulating lymphocyte pool is the only compartment readily accessible for monitoring specific T-cell responses, although it may not be the most relevant in terms of establishing correlations with clinical tumor response. An important limitation at present relates to the sensitivity of these assays which is in the order of approximately 1 in 10,000 CD8$^+$ T lymphocytes. It is becoming clear that the frequency of tumor antigen-specific CD8 T cells is frequently below this limit of detection, even after repeated vaccination. Hence the common practice in monitoring current clinical trials of vaccination of introducing steps of in vitro antigen-driven amplification of T cells before measurement of multimer$^+$ T cells, or of specific cytokine release assay. Thus, most of the valuable information on in vivo frequency and functional differentiation of T cells upon vaccination is lost. A solution to this problem has been devised recently in a study quantitating the frequency of MAGE-3.A1–specific T cells before and after vaccination [12, 13]. It involves two or three rounds of in vitro stimulation with peptide in multiple single cell dose microcultures combined with detection of expanding specific T cells in individual microcultures by flow cytometry with multimers. This method has been used to estimate the frequency of antigen-specific T cells, although it is not a complete limiting dilution analysis. While this approach is quite labor intensive, it allows us to monitor T-cell activation at low T-cell frequencies. Hopefully, the learning process on vaccine optimization will lead to the consistent induction of high frequency systemic CTL responses in the future so that their direct and accurate monitoring with the modern tools described above will be possible.

We have used fluorescent HLA-A2 multimers carrying several of either cancer/testis or melanocyte/melanoma differentiation antigenic peptides to assess the naturally acquired CTL response to various tumor antigens. To establish baseline values of frequency of tumor antigen specific T cells in cancer patients, we measured the numbers of multimer$^+$ T cells in

peripheral blood lymphocytes (PBMCs) in series of HLA-A2 metastatic melanoma patients. The experimentally determined sensitivity of multimers varies from 1 in 2,000 to 1 in 10,000 $CD8^+$ PBMCs (0.05–0.01%). Memory T cells directed against dominant viral epitopes such as influenza matrix 58–66, Epstein-Barr virus BMLF 280–288, or CMV pp65 495–503 are readily detectable above this detection limit in a high proportion of individuals. In contrast, generally, $multimer^+$ T cells for gp100 [14], tyrosinase [15], MAGE-A10 [16], NY-ESO-1 [17], or SSX-2 [18] can not be detected directly ex vivo, but only after expansion by repeated in vitro stimulation with the antigenic peptide on appropriate antigen-presenting cells. Some exceptions involving the tyrosinase antigen have been reported in advanced metastatic melanoma patients. In one case, the $multimer^+$ T cells were functionally anergic [8], whereas in the second report they were functionally active ex vivo [9].

In marked contrast to most tumor antigen specific T cells which are not detectable ex vivo by multimer-assisted flow cytometry, an abundant repertoire of HLA-A2/Melan-A/MART-1 $multimer^+$ T cells was identified. Indeed, we were able to trace the origin of this repertoire to a particularly efficient positive selection in the human thymus [19]. It appears that the large numbers of Melan-A/MART-1 $multimer^+$ T cells are maintained throughout the adult life of healthy individuals at high numbers (on average approximately 1 in 1,400 $CD8^+$ T cells) in a functionally naïve state. Thus, T cells specific for the HLA-A2-Melan-A/MART-1 antigen are easily detectable by multimer staining directly ex vivo [20, 21]. At some time during melanoma tumor progression some of these T cells are activated and recruited to the tumor site [22]. In PBMCs from melanoma patients, their mean frequency was not significantly different from that measured in healthy individuals but variable proportions of these cells exhibited an activated/memory phenotype. Remarkably, the mean frequency of Melan-A/MART-1 $multimer^+$ T cells found in fresh metastatic lymph node cell suspensions was of 1 in 30 in two thirds of HLA-A2 melanoma patients, that is 25-fold higher than in the peripheral blood. Moreover, close to 100% of these cells exhibited an activated/memory phenotype, characterized as $CD45RA^{low}$ and $CCR7^-$. Thus, the Melan-A/MART-1 constitutes an excellent model antigen in humans to monitor the naturally acquired antitumoral T-cell responses as well as to precisely monitor the response elicited by various strategies of vaccination.

We have also assessed the Melan-A/MART-1–specific CD8 T cell response to experimental vaccination [23]. We conducted phase I clinical trials in advanced melanoma patients using Melan-A/MART-1 peptide, either the wild-type peptide or a modified peptide analogue, alone or in combination with immunological adjuvants. Peptides (100 μg/dose) were injected at 4-week intervals, either alone or mixed with one of two adjuvants approved for human use: the AS02 (provided by GlaxoSmithKline, Belgium), which consists of the saponin QS21, monophosphoryl lipid A, and an oil-in-water emulsion; and Montanide (Seppic, Paris, France) which contains the mineral oil Drakeol and anhydro mannitol octadecanoate. To date we have completed the analysis of 49 vaccinated patients. All patients tolerated well the various vaccine formulations. Measurement of the frequency of A2/Melan-A/MART-1 $multimer^+$ $CD8^+$ lymphocytes in blood samples obtained before and after peptide vaccination showed increases following vaccination in 13 patients. One patient, who received the wild-type Melan-A/MART-1 peptide with the AS02 adjuvant, had a 23-fold expansion and reached a plateau frequency of A2/Melan-A/MART-1 $multimer^+$ lymphocytes of about 2% of the circulating $CD8^+$ T-cell compartment. The remaining patients had more modest expansions ranging between twofold and twelvefold the prevaccination $multimer^+$ T-cell frequency. Interestingly, the latter were in the group immunized with peptide and Montanide. In fact, 12 out of 17 patients in this group had a detectable specific CTL response to peptide inoculation. Other clinical trials of vaccination with Melan-A/MART-1 and gp100 peptides have also shown the efficiency of Montanide as an adjuvant for CTL induction in melanoma patients [24, 25]. We also assessed the evolution of the cell surface phenotype of A2/Melan-A/MART-1 $multimer^+$ T lymphocytes over time after peptide vaccination. Interestingly, we could identify significant phenotype shifts in Melan-A/MART-1–specific T cells in all five patients with detectable frequency increases. This consisted of up-regulation of HLA-DR and 2B4 as well as down-regulation of CD45RA, CCR7, and CD27 in variable proportions of $multimer^+$ T lymphocytes [23].

A detailed analysis of the T-cell response in the patient who had the most marked increase in A2/Melan-A/MART-1 $multimer^+$ T cells following immunization revealed interesting features that may provide valuable hints to future monitoring of cancer vaccines: (1) As assessed by ELISPOT and intracellular staining, the absolute number of Melan-A/MART-1–specific T cells able to secrete IFN-γ increased >50-fold upon vaccination. This represented about 40% of the specific cells that could rapidly secrete IFN-γ, while no IL-4 production could be detected under the same experimental conditions [26]. When tested directly ex vivo after sorting from the postimmunization blood sample, Melan-A/MART-1–specific cells were weakly cytolytic but became highly active after in vitro restimulation. Together, these results are in agreement with the effector cell surface phenotype of the specific cells. They indicate that large numbers of functionally active tumor-specific $CD8^+$ T cells could be obtained and maintained at high levels after in vivo activation by repeated peptide-based vaccination. (2) Importantly, we documented a distinct increase in the functional avidity of antigen recognition and in tumor reactivity in the postimmune Melan-A/MART-1–specific populations as compared to the preimmune specific cells [27]. Improved antigen recognition correlated with an increase in the half-life of A2/peptide

multimer interaction with the TCRs as assessed by kinetic analysis of A2/Melan-A/MART-1 multimer staining decay. (3) Ex vivo analysis of the TCR Vβ repertoire used by Melan-A/MART-1–specific $CD8^+$ T cells at different time points during vaccination revealed that the response was the result of asynchronous expansion of several independent T-cell clones. Some of them could also be identified in TILs recovered from a metastatic tumor site excised during the vaccination period [27]. These data thus provide further evidence for a tumor peptide-driven immune response resulting in the selection of high avidity T-cell clones of increased tumor reactivity that appear to evolve independently within oligoclonal populations of specific T cells. (4) Some subcutaneous and lymph node metastases regressed completely and even a lung metastasis underwent partial objective regression, while the remaining subcutaneous and visceral metastases were stabilized for a period of 1 year. Together, these results raise the possibility that a strong and systemic specific T-cell response may exert potent antitumor effects.

Concluding remarks

The 1980s were characterized by efforts to understand the role of T cell—mediated immunity in tumor recognition and culminated with the cloning of the first CTL-defined human tumor antigen in 1991 [28]. One decade later, a relatively large volume of clinical results with a variety of cancer vaccines based on molecularly defined tumor antigens has been acquired. Despite the relative success of vaccination at inducing specific immunity, clinical efficacy remains marginal. Thus, there are important hurdles to overcome to attain a high level of induced immunity in every vaccinated patient and, more importantly, good clinical efficacy. The number of parameters in the process of vaccine optimization is large and imposes the need to carry out a large number of exploratory clinical studies to discriminate between multiple possible vaccination strategies [29]. To quote only one recent example of the complexities involved in even the most simple form of vaccine delivery, the peptides, it was shown that the use of a 11 amino acid peptide instead of the exact 9 amino acid antigenic peptide led to the induction of a peptide-specific response biased for immunodominant epitope that is not expressed naturally by the tumor [30, 57]. Although preclinical studies in transgenic HLA mice, or even in nonhuman primates, might be of help, the most direct way remains to perform carefully planned phase I clinical trials of vaccination with defined tumor antigens. Only the systematic and standardized quantitative and qualitative monitoring of antigen-specific T-cell responses will allow us to make rapid progress towards optimal vaccination procedures in humans.

Great progress in monitoring antigen-specific class I–restricted T-cell responses in humans has been made in parallel with progress in human tumor immunology. Nevertheless, monitoring of the local immune responses is still at its early stages. Recent studies have shown the feasibility of using fluorescent multimers in fresh or frozen tissue sections [31, 32, 33, 34]. Moreover, the limited amount of tumor material requires the adaptation of sensitive molecular tools to monitor gene expression in different clinical situations and identify potential correlates of immune responsiveness [35].

Multiple mechanisms of tumor escape from immune pressure have been well documented in recent years [36]. In fact, tumor escape has been identified as one of the major stumbling blocks in the search for clinically effective cancer vaccines [37]. Strategies targeting multiple tumor antigens and including T-cell help should help in coping with this problem. In this regard, the identification of tumor antigens targeted by CD4 T cells is progressing at a fast pace [38, 39]. MHC class II–peptide multimers are becoming available and should facilitate the monitoring of specific CD4 T-cell responses [40, 41]. Finally, vaccination at the early stages of cancer progression may greatly minimize the chances for tumors to escape.

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