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## **Mechanistic studies on long peptide based vaccins for the use in cancer therapy.**

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Design and development of  
synthetic peptide vaccines

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## **Design and development of synthetic peptide vaccines: past, present, and future.**

**Abstract.** Synthetic peptide vaccines aiming at the induction of a protective CD8<sup>+</sup> T cell response against infectious or malignant diseases are widely used in the clinic but despite their success in animal models they yet do not live up to their promise in humans. This review assesses the development of synthetic peptide vaccines, weighs it against the immunological concepts that meanwhile have emerged, and identifies the key issues that play a role in failure or success of a synthetic peptide vaccine. The current state-of-the-art peptide vaccine is a complete synthetic inflammatory product that is ingested by professional antigen presenting cells and stimulates both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.



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## SYNTHETIC PEPTIDE VACCINES: IMMUNITY VERSUS TOLERANCE

In the 1950s the host's immune system was shown to reject tumors following the recognition of tumor-associated Ag (TAA) (1-4). Subsequently, Zinkernagel and Doherty discovered in 1974, that the recognition of target cells by CD8<sup>+</sup> T cells was restricted by the Major Histocompatibility Class (MHC) I molecules (5). Townsend et al. then revealed that CD8<sup>+</sup> T cells were able to sense short linear peptide sequences of 11-16 amino acid (a.a.) in length, generated by proteolytic degradation of the target antigens that were presented in the context of MHC class I molecules (6-8). Subsequently, the group of Rammensee and colleagues revealed that the exact MHC class I binding peptides (CD8<sup>+</sup> T cell epitopes, Fig. 1A) recognized by CD8<sup>+</sup> T cells were even smaller peptides - 8 amino acids (a.a.) (9-11) - and that these peptides contained specific amino acids that anchored the peptide in the pockets of the MHC class I molecule (12). This knowledge resulted in the rapid identification of CD8<sup>+</sup> T cell epitopes in both mice (13-16) and human beings (12) and in exploration of the use of peptide vaccines against multiple diseases.

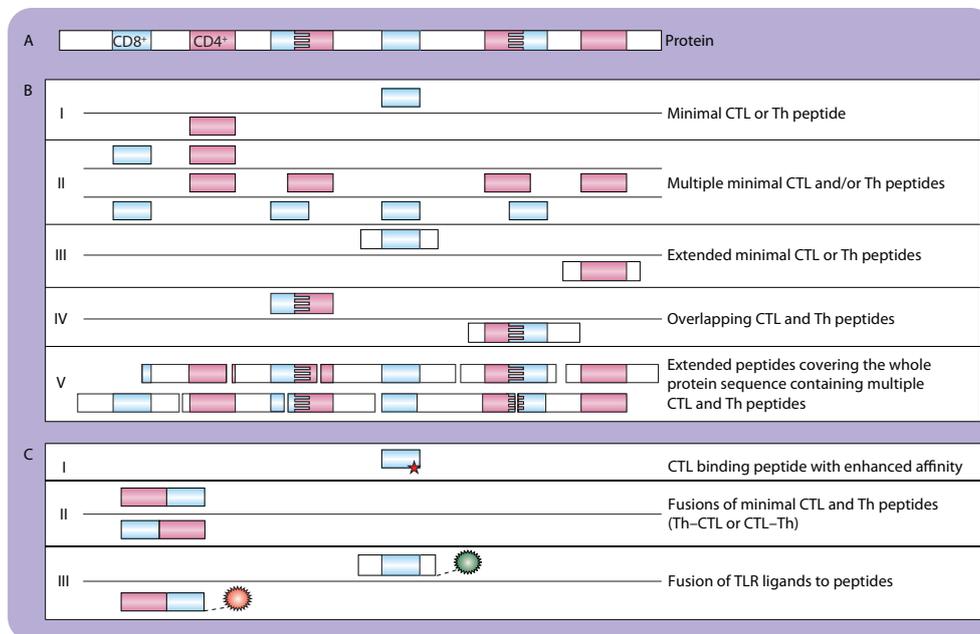
The first peptide vaccine able to induce a Cytotoxic T lymphocyte (CTL) response *in vivo*, was reported by Aichele et al. (17) who injected mice with a peptide encoded by lymphocytic choriomeningitis virus (LCMV) (Fig. 1B). Peptide vaccination was also shown to induce a protective CTL response against lethal challenges with live viruses, such as LCMV (18) and Sendai virus (19), against a lethal challenge with Human Papilloma Virus (HPV) type 16 positive tumor cells (20) or methylcholanthrene-induced sarcomas (21), and was shown to enhance the life-span of mice in a spontaneous lung carcinoma metastasis model (22) (Fig. 1B). However, not all peptide vaccines induced strong protective CTL responses. For instance, vaccination with peptides from the circumsporozoite protein from malaria (23), or GP100 (24) induced low levels of CTL responses while vaccination with Her2/neu peptides in patients induced only short-lived CD8<sup>+</sup> T cell responses (25). In contrast, some peptide vaccines did not induce protective CTL responses (26-28) or even enhanced tumor cell growth due to the induction of CD8<sup>+</sup> T cell tolerance (29,30). Despite these opposing outcomes, many synthetic peptide-based clinical trials have been initiated in cancer patients with disappointing immunological and clinical responses (reviewed in (31,32)). In this review we have explored the short-comings of these peptide-based vaccines and provide a basis for the rational design of improved peptide vaccines that are able to induce strong and sustained T cell responses. Since a complete overview of the results obtained by peptide vaccines in patients has recently been published by Mocellin et al. (31), we only refer to specific examples in this review.



## HOW TO ENHANCE THE CD8<sup>+</sup> T CELL RESPONSES

### Increasing the peptide affinity.

Many (self) minimal CTL peptides have relatively low binding affinity for the MHC class I complex and, therefore, are not able to induce a strong CD8<sup>+</sup> T cell response. The affinity of the peptide (33-35) and the dissociation constant of the peptide (36) determine the time before the peptide:MHC complex is displaced from the cell surface. Site directed substitution of specific amino acid positions in these peptides (Fig. 1C, I) can therefore induce more stable peptide:MHC class I complexes and thereby enhance the immunogenicity of these



**Figure 1. Design of peptide-based vaccines; choosing and designing the right peptide(s).** A. Definition of CD8<sup>+</sup> and CD4<sup>+</sup> T cell epitopes. In a given protein sequence there can be multiple CD8<sup>+</sup> (blue) and CD4<sup>+</sup> (purple) T cell epitopes present that can bind to different MHC/HLA class I and II molecules, respectively. These CD8<sup>+</sup> and CD4<sup>+</sup> T cell epitopes can be located at distinct positions in the protein sequence, or could be (partially) overlapping; striped blue/purple bars. B. Peptide vaccines can come in many flavors, for instance: I) minimal CTL or Th peptides; II) multiple minimal CTL and/or Th peptides; III) minimal CTL and Th peptides that are N- and C-terminally extended; IV) single peptides that contain both a CTL and a Th epitope that (partially) overlap; or V) a set of overlapping peptides that cover the whole protein sequence, containing all possible CD8<sup>+</sup> and CD4<sup>+</sup> epitopes of the protein. C. Engineered peptide vaccines with enhanced immunogenicity. In order to enhance the immunogenicity of a peptide vaccine several strategies can be undertaken: I) the affinity of a MHC/HLA class I binding peptide can be altered/enhanced by changing certain amino acids (red star); II) minimal CTL and Th peptides can be physically linked to form long Th-CTL or CTL-Th fusion peptides; or III) different forms of peptides can be conjugated to TLR ligands.



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peptides (33,37-39). As a result, injection of these modified peptides could confer protection against a subsequent lethal tumor challenge in mice (40,41) as well as increase the magnitude of the CD8<sup>+</sup> T cell response in melanoma patients (42,43).

Another approach to enhance the immunogenicity of MHC class I restricted peptides is to design a super-agonist peptide able to interact more efficiently with the T cell receptor of CD8<sup>+</sup> T cells (Fig. 1C, I), as demonstrated with the Melan-A/Mart-1<sub>27-35</sub> epitope (44). Site directed substitution of certain a.a. in the minimal CTL peptides could therefore be a genuine strategy to enhance the magnitude of peptide vaccine induced T cell responses. However, in some cases alteration of peptides resulted in the induction of T cell responses with TCRs not able to cross-react with the wild type peptide (45,46), indicating the limitations in applying such a strategy.

#### **Inclusion of CD4<sup>+</sup> T cell peptide-epitopes; a helping hand for the Cytotoxic T Lymphocyte.**

Central to the induction of strong and sustained CTL immunity is the presentation of minimal CTL peptides by matured DC which endow the responding CD8<sup>+</sup> T cells with the necessary signals that allow them to become functionally active (47). In addition, CD8<sup>+</sup> CTL require the presence of cytokines such as IL-2 that support them to rapidly expand. Lack of DC maturation or supporting cytokines may result in a vaccine-induced CD8<sup>+</sup> T cell response that is transient and weak, even when peptides with strong MHC binding affinity are used. A proper way to increase the magnitude and long-term efficacy of the CD8<sup>+</sup> T cell response is through the provision of help by CD4<sup>+</sup> T helper cells (Fig. 1B).

The helping role of CD4<sup>+</sup> T cells is versatile. Upon activation, the CD4<sup>+</sup> T cell can secrete the cytokine IL-2 (48), which is an important growth factor for (CD8<sup>+</sup>) T cells; CD4<sup>+</sup> T cells can activate the DC via CD40-CD40L interactions (49-51) that can subsequently license the CD8<sup>+</sup> T cell to kill its target cell and CD4<sup>+</sup> T cells can program CD8<sup>+</sup> T cells via the Antigen Presenting Cell (APC) to become memory cells (52).

This important role for the CD4<sup>+</sup> T cell in the induction of a protective CD8<sup>+</sup> T cell response by peptide vaccination against LCMV became apparent after it was realized that the peptide vaccine used by Aichele et al. (17) comprised both a CD8<sup>+</sup> T cell epitope and a CD4<sup>+</sup> T cell epitope (53) (Fig. 1B, IV). *In vivo* depletion of the CD4<sup>+</sup> T cell population almost completely abrogated the vaccine-induced protective effect against LCMV (53). In addition, CD4<sup>+</sup> T-cell help was shown to be indispensable for the induction of long lasting immunity against tumors. Eradication of MHC class II-negative FBL erythroleukemia cells was only observed when the mice were injected with a vaccine that only contained a tumor-specific Th peptide (54) (Fig. 1B, I). Indeed vaccination with an exclusive Th peptide protected mice against



MHC class II-negative virus induced tumors (Fig. 1B, I), but optimal protection was achieved by vaccination with both a minimal Th peptide and a dominant minimal CTL peptide (28) (Fig. 1B, II). This principle of providing help was endorsed in a phase I clinical trial in which patients were vaccinated with minimal CTL peptides of melanoma-associated tyrosinase antigen. Tyrosinase peptide specific IFN $\gamma$ -producing T cells were detected only if the patients simultaneously received the non-specific helper protein keyhole limpet hemocyanin (KLH) in addition to the minimal tyrosinase CTL peptide vaccine (55).

Further improvements in peptide vaccines were achieved by directly linking the minimal Th peptide to the minimal CTL peptide to form a single linear hybrid peptide [PbCS(57-70)-(251260)] (Fig. 1C, II) showed enhanced CTL responses in a mouse model for malaria (23). Similar observations were reported for peptide vaccines aiming at the induction of Human Immunodeficiency Virus-specific CTL (56) and Herpes Simplex Virus-specific CTL (57). However, not all the combinations of minimal Th and CTL peptide linkage showed enhanced reactivity. For instance, in a phase I clinical trial with stage IV patients, a gp100 specific T cell response was only observed in patients that had received the minimal CTL peptide gp100 and not in patient vaccinated with the gp100 minimal peptide that was C-terminally extended with a Th peptide derived from tetanus (58). The reason for the absence of gp100 specific T cells could be the additional amino acid sequence located at the C-terminus of the CTL epitope. Changing of the natural occurring C-terminal amino acid sequence can change the processing by the proteasome and thus liberation of the CTL epitope (59,60).

Interestingly, a number of peptide vaccines able to induce strong CD8<sup>+</sup> T cell responses, not only comprised the minimal CD8<sup>+</sup> T-cell epitope but also a CD4<sup>+</sup> T cell epitope, e.g. LCMV (53), HPV (61), and mutant p53 (21) (Fig. 1B, IV). Co-localization of CD4<sup>+</sup> and CD8<sup>+</sup> epitopes has also been described in proteins that are target antigens in human beings, e.g. NY-ESO (62), Synovial Sarcoma X-2 breakpoint protein (63), and Mart-1/Melan-A (64). An elegant approach was undertaken by Knutson et al. who identified Her2/neu CD4<sup>+</sup> T cell epitopes that comprised a (HLA-A2<sup>+</sup>) CD8<sup>+</sup> T cell epitope within the same peptide sequence (65) (Fig. 1B, IV). In contrast to their clinical trial in which only a CD8<sup>+</sup> T-cell epitope was used (25), vaccination with the peptides, comprising both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell epitopes, resulted in the induction of long-lived Her2/neu specific CD8<sup>+</sup> T cell responses. Thus, a simple approach to induce strong and long-term CD8<sup>+</sup> T cell reactivity is the inclusion of CD4<sup>+</sup> T cell epitopes in peptide vaccines.



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### **Antigen-related or non-related CD4<sup>+</sup> T cell help.**

Addition of a minimal Th peptide enhanced the capacity of peptide vaccines to induce strong malaria-specific CTL responses (23,66) and protected mice against a subsequent tumor challenge (28,67,68). As yet it is not clear whether the CD4<sup>+</sup> T cell epitope that is incorporated in the vaccine should be derived from the same (tumor) Ag or pathogen as the CD8<sup>+</sup> T cell epitope. It has been argued that for optimal results both epitopes should be encoded in the same pathogen or tumor (28) (Fig. 1B, II, IV, V), while others have shown that this is irrelevant (23,55,67,69). This issue can only be solved when the intrinsic properties of CD4<sup>+</sup> T-cell epitopes (Fig. 1A) - i.e. binding affinity or antigenic origin - are taken into account. It can be envisaged that CD4<sup>+</sup> (viral) T cell peptides with high binding affinity for the MHC class II molecule will be more helpful to a developing CD8<sup>+</sup> T cell response than the use of CD4<sup>+</sup> T cell peptides which have a low binding affinity for MHC class II, as was the case for the non-related MHC class II I-Ab-restricted epitope of Ovalbumin (28). Similarly, CD4<sup>+</sup> T cell epitopes derived from proteins to which partial tolerance exist may be less powerful with respect to the induction of a strong helper T cell response (70).

Help can also be provided in the form of an agonistic CD40 Ab that triggers the CD40 receptor on APC (49). Using CD40 agonistic Ab, a prophylactic vaccine consisting of the minimal CTL peptide of HPV16 E7 protein could be converted in to a vaccine with therapeutic anti-tumor potency (71) (Fig. 2, I). Furthermore, the use of agonistic CD40 Ab allowed eradication CD40 negative tumors in tumor-bearing mice (72). This depended on the activation of tumor-antigen presenting CD11c<sup>+</sup> APC in the draining Lymph Node (LN) (73). However, this type of "help" requires complete tumor or virus eradication, because it is associated with an acute wave of effector CTLs. In case of chronic disease, additional injections (of aCD40 Ab) are required.

This latter argument also applies to the use of non-related CD4<sup>+</sup> T cell epitopes in peptide vaccines. Although such CD4<sup>+</sup> T cell epitopes can effectively induce a CD4<sup>+</sup> T cell response that helps the development of an acute wave of CTL, this CD4<sup>+</sup> T cell response is rather useless in re-activating the CTL response upon secondary challenge or to sustain CTL responses in case of chronic infection/diseases, simply because the cognate Ag for these CD4<sup>+</sup> T cells is lacking. Provision of both the minimal (non-related) Th and (related) CTL peptides in IFA - that induces long-term Ag presentation of both peptides simultaneously in the dLN (74) - might however, sustain APC activation and thereby CTL reactivity and might to some extent circumvent this problem. Note, however that the vaccination with Ag-related minimal Th peptides will ensure reactivation of these Ag-specific CD4<sup>+</sup> T cells, that subsequently provide help to the Ag-specific CD8<sup>+</sup> T-cell response upon re-challenge.



I	 APC- and T-cell-activating Abs (aCD40, OX-40, 4-1-BB, aCTLA-4, aPD-L1)	Abs that enhances T-cell activation, expansion and survival; or activate APCs in vivo
II	 Cytokines/T-cell growth hormones  Cytokine complexed to specific Abs  Cytokine complexed to soluble receptors	Enhances expansion, effector function, and survival of T cells. Complexation to specific Abs or soluble receptors reduces the in vivo toxicity
III	 Cyclophosphamide  IL-2 linked to diphtheria toxin (DAB389IL-2)	Depletion of Tregs to remove inhibitory mechanisms and to enhance vaccine efficacy
IV	 Chemotherapy, $\gamma$ -irradiation TLR ligand-containing cream	Induction of local inflammation to enhance T-cell infiltration and T-cell function

**Figure 2. Additional immune interventions to enhance vaccine efficacy.** In order to enhance the immunogenicity and efficacy of a peptide vaccine additional immune interventions can be applied. Ab: Antibody; APC; Antigen-presenting cell; TLR Toll-like receptor; Treg; T-regulatory cell.

### Length of the peptide.

Unexpectedly, the length of the peptide used for vaccination strongly influences the magnitude of the induced CD8<sup>+</sup> T cell response (Fig. 1B, III-IV). In a head-to-head comparison, a 35 amino acid long peptide containing the HPV16 E7<sub>49-57</sub> CD8<sup>+</sup> T cell epitope induced a robust CD8<sup>+</sup> T cell response capable of eradicating an established tumor, while the minimal CTL peptide (9 a.a. long) could not (75). One of the underlying mechanisms is that extension of these minimal CTL peptides to a longer peptide, increased the duration of Ag presentation *in vivo* and thereby enhanced the magnitude of the CD8<sup>+</sup> T cell response and reviewed in (reviewed in (76) and C).

Additionally, the presentation of the CTL epitopes from the extended peptides requires Ag processing by professional APC, whereas the minimal CTL peptide can bind exogenously to MHC class I molecules. This might also explain why many of the first reported, so called “short synthetic peptides” performed so well. These “short synthetic peptides” were indeed relatively short when compared to whole antigens, but still were not the exact MHC class I binding peptides that - based on these animal models - are nowadays used in vaccine trials in humans. Actually, the vaccines used in these animal models could reach up to 27 amino acids in length (17-20,77-80) (Fig. 1B, III-IV) and, as such, should be qualified as Ag-processing dependent peptides that are more immunogenic than their exact MHC class I binding counterparts (75).



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### Using adjuvants to enhance immune reactivity.

The most devastating signal a T cell can receive is that through interaction of its T-cell receptor and a MHC-peptide complex presented on a non-activated and non-matured APC. T cell activation in the absence of co-stimulatory molecules or pro-inflammatory cytokines will generally result in the induction of tolerance (reviewed in (81)). The main hallmark of good vaccines is that they closely mimic the most successful natural triggers of DC activation. Therefore peptide vaccines have to be formulated with different kinds of adjuvants that enhance the magnitude, survival and polarization of the vaccine-induced T cells.

A very common vehicle and adjuvant for vaccination is oil-in-water emulsion (reviewed in (82)). The best known are: Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), and Montanide ISA-51. Besides CFA – that contains heat killed *Mycobacterium tuberculosis* – the other two adjuvants only contain mineral oil and their adjuvant effect is based on the formation of a depot from which Ag is slowly released into the system. Another promising adjuvant is Immuno Stimulatory Complexes (ISCOMs) ((83) and reviewed in (84)). ISCOMs are cage-like structures that are comprised of antigen, cholesterol, phospholipid, and saponin that targets the antigen and adjuvant components of the vaccine to both the endosomal and cytosolic pathways for antigen presentation.

Other ways to strongly activate DC are the use of Toll Like Receptor (TLR) ligands. These ligands mimic certain structures commonly found on the outside and inside of pathogens that can deliver danger signals upon binding to the TLR at the surface or inside the APC. Examples of the most common naturally occurring danger signals are: pathogen-derived RNA or DNA, lipoproteins/peptides, lipopolysaccharide (LPS), and peptidoglycan (reviewed in (85)). Inclusions of synthetic analogs of these TLR ligands into vaccines provide an ultimate way to enhance vaccine induced T cell responses via the activation of TLR on professional APC. So far many different TLR ligands have been exploited in vaccine studies in mice ((75,86) and Chapter 4+5). In human beings, monophosphoryl lipid A (a detoxified form of LPS) - that triggers TLR4 - was proven to be safe as a clinical vaccine adjuvant in combination with a recombinant protein vaccine against malaria (87). The use of CpG 7909 – a TLR9 ligand – in combination with a Melan-A minimal CTL peptide in IFA not only enhanced the magnitude of Melan-A specific CD8<sup>+</sup> T cells compared to the minimal CTL peptide vaccine without CpG, it also changed the phenotype of these CD8<sup>+</sup> T cells from IFN $\gamma$  producing cells to IFN $\gamma$  producing cells that were also granzyme and perforin positive (88). Direct coupling of the TLR ligands to Ag forms a next generation of vaccines (Fig. 1C, III). A pioneer study by Rammensee showed that the use of peptide linked to lipopeptides induced the same high affinity T cells as live influenza virus (89). Others have shown that coupling enhances Ag uptake by professional APC ((90) and Chapter 5), enhances Ab and T cell



responses (91,92) and induces a more vigorous and protective T cell response to pathogens, with similar efficacy as a live vaccines (93). While the use of TLR ligands, and especially multiple TLR ligands (94,95), generally provides a potent APC stimulating environment needed for the induction of effector T cell responses, they may also unleash unwanted T-cell responses. For instance, in mice the use of TLR2 ligand (Pam3Cys) induces proliferation of Treg cells (96) and systemic application of CpG elicits potent regulatory responses by acting on a discrete, minor population of splenic DCs (97).

### **PHARMACOKINETICS OF PEPTIDE VACCINES DETERMINE OUTCOME**

Of all the vaccinations with minimal CTL peptides (Fig. 1B), many resulted in the induction of a detectable but weak CD8<sup>+</sup> T cell response and some peptide vaccines were even reported to induce T cell tolerance. Multiple (systemic high dose) vaccinations with the minimal CTL peptide GP<sub>33-41</sub> of LCMV in IFA resulted in CD8<sup>+</sup> T cell tolerance (27) or deletion of GP<sub>33-41</sub> specific transgenic CD8<sup>+</sup> T cells (26). This induction of CD8<sup>+</sup> T cell tolerance has been attributed to the multiple high doses of peptide, which likely over-activated/stimulated the responding CD8<sup>+</sup> T cells and subsequently induced apoptosis (27).

However, Toes et al. reported in an adenovirus tumor model that already a single injection with a low dose of the strong MHC class I binding CD8<sup>+</sup> T cell peptide-epitopes of adenovirus (Ad5E1A or Ad5E1B) in IFA tolerized adenovirus-specific CD8<sup>+</sup> T cells and enhanced tumor cell outgrowth (29,30). This result is not restricted to vaccination with these Ad5E1A or Ad5E1B minimal CTL peptides, as this also occurred after vaccination with the highly immunogenic Ovalbumin<sub>257-264</sub> minimal CTL peptide (Chapter 2).

#### **Mechanism of peptide vaccine induced tolerance.**

The tolerogenic feature of Ad5E1A has been attributed to the fact that the Ad5E1A minimal CTL peptide very rapidly distributes throughout the whole body in contrast to, for instance an immunogenic minimal CTL peptide, like HPV16 E7 (98). Of note, the Ad5E1A minimal CTL peptide displays a very strong binding affinity to its cognate MHC class I molecule, whereas the HPV16 E7 minimal CTL peptide displays only intermediate affinity to the MHC class I molecule (33). We realized that the injection of these high affinity minimal MHC class I binding peptides resulted in the binding to cell surface expressed MHC class I molecules of passenger lymphocytes in the dLN and showed that these lymphocytes export the peptide to more distant lymph nodes (Chapter 3). The slow release and thereby long duration of Ag presentation induced by the IFA adjuvant – more than 100 days (74) – in combination with



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minimal CTL peptides presentation in non-inflammatory LN by non-professional APC, will provide an optimal environment to induce CD8<sup>+</sup> T cell tolerance (99,100), or the deletion of specific CD8<sup>+</sup> CTL when peptide vaccine-activated CD8<sup>+</sup> T-cells present the minimal CTL peptide themselves- fratricide (101). These data suggest that the injection of modified minimal CTL peptides displaying a strongly increased peptide binding affinity for MHC class I molecules in an oil-in-water adjuvant such as IFA or Montanide ISA51, might work counterproductive.

#### **Prevention of peptide vaccine induced tolerance.**

The studies described above indicate that the pharmacokinetics of the peptides used for vaccination are important to evaluate the capacity of a peptide vaccine to induce an effective immune response. In addition, help in the form of agonistic CD40 Ab (Figure 2,I) initially enhanced the magnitude of the Ad5E1A-specific (102) CD8<sup>+</sup> T cell responses induced by peptide in IFA vaccines, however, this treatment did not prevent the tolerization of the responding CD8<sup>+</sup> T cells at later stages (74). While the aCD40 antibody will be cleared rapidly from the system and as such is not able to activate the APC, the CD8<sup>+</sup> T cell continuously receives signals by the minimal CTL peptide leaking out of the IFA depot for more than 100 days (74) presented by non-activated APC. Importantly, the provision of continuous help by co-injection of a minimal Th peptide (Fig. 1B, II) in IFA, rescues CD8<sup>+</sup> T cells from tolerance, indicating that CD4<sup>+</sup> T cell help is not only important in enhancing the CD8<sup>+</sup> T cell response as has been reported previously (23), but also in preventing the induction of CD8<sup>+</sup> T cell tolerance (Chapter 2). These results teach us that both the APC activating signals and the T-cell activating signals (peptide) should be matched both in time and location.

#### **MULTI-EPITOPE VACCINES TO PREVENT IMMUNE ESCAPE**

Many vaccination trials are performed with only a single minimal CTL peptide designed to fit only one HLA class I molecule and are therefore not widely applicable to many patients of different genetic backgrounds (Fig. 1B, I). Such a strategy not only narrows down the overall force that can be undertaken against an infected/transformed cell but also constrains the efficacy of the vaccines in the case of Ag loss variants or specific down regulation of HLA class I molecules by the target cell.

This was exemplified by the sequential HLA and Ag loss variants of subsequent metastases of melanoma in a patient reported by Yamshchikov et al. (103). The first escape-variant showed selective loss of three HLA class I molecules. The second metastasis displayed the specific down-regulation of a HLA molecule involved in the presentation of a Mart-1



specific peptide, while still presenting a tyrosinase-derived epitope. Accordingly, Lehmann et al. reported that CTL lines that recognized the first metastasis did not cross react with the second metastasis, due to the selective loss of the HLA molecules to which the CTL lines were restricted (104). New CTL clones which specifically recognized the second metastasis were directed to a different HLA class I molecule and to a different antigen compared to the first metastasis.

#### **Multiple peptide-epitopes vaccines.**

The most effective strategy for vaccination is the use of multiple CTL and Th epitopes of a given protein Ag so that they can be presented by many different HLA (Class I and II) molecules, reducing the chance of outgrowth of Ag and/or HLA class I loss variants. This can be achieved by vaccination with the whole Ag as a vaccine; either by overlapping long peptides that span the whole protein (Fig. 1B, V); or by using recombinant or synthetic proteins (Fig. 1A). Furthermore, attacking the cell via multiple Ag, would further enhance the strength and as such the success of the vaccine.

For instance, the therapeutic use of overlapping peptides (27-34 a.a. long, Fig. 1B, V) spanning the entire Cottontail Rabbit Papilloma Virus (CRPV) E6 and E7 proteins was reported to induce regression of established warts induced by CRPV and a reduction of the CRPV viral load (105). A similar vaccine consisting of overlapping peptides (27-34 a.a.) spanning the sequence of HPV16 E6 and E7 induces CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in cervical cancer patients (Melief and Van der Burg, unpublished data). Also longer peptides, ranging from 44-182 a.a. in length were effective in inducing strong Ab and T cell responses in mouse and human malaria model systems (106-110). In addition, linking of two long HPV16 E7 peptides to create the synthetic protein (98 a.a. long) of HPV16 E7 was shown to be highly effective in clearing an established tumor (111).

Besides the use of multiple antigens to target virally infected cells or tumor cells, the choice of proteins may contribute to the clinical success of vaccination. It is likely that proteins less susceptible to mutations (structural proteins of HIV) or proteins that are needed to maintain the oncogenic form of the cell would provide better target antigens. Examples would be anti-apoptotic proteins such as survivin (112), bcl-2 (113), bcl-xL (114), and Mcl-1 (115). Accordingly, telomerase reverse transcriptase (hTERT) (116) or proteins that are involved in resistance to chemotherapeutic agents such as TRAG-3 (taxol resistance gene) (117) might constitute appropriate target antigens for T-cells to attack malignant cells.



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## EXPERT COMMENTARY

Minimal CTL peptide vaccinations are widely used against multiple forms of cancer and other diseases. However, these peptide-based vaccines have so far induced little to no clinical or immunological responses (reviewed in (31,32)). We are convinced that the translation of peptide vaccination from early pre-clinical mouse studies (17-20,27,77-80,118) to human clinical studies was flawed by an imperfect understanding of the mechanisms that play a role in successful peptide vaccination. First, while studies in human subjects almost always explore the efficacy of vaccines consisting of the exact HLA class I binding peptides (Fig. 1B, I), most of the groundbreaking studies which showed that “short synthetic peptide” vaccines could be effective in pre-clinical mouse studies, used peptides that were not exact MHC class I binding peptides. In general, the peptides were much longer and could reach up to 27 a.a. in length (17-20,77-80) (Fig. 1B, III-IV) and are therefore likely to be retained in the local draining lymph node where they are processed and presented by professional APC to CD8<sup>+</sup> T cells ((75) and reviewed in (76)). Additionally, some of the “short synthetic peptides” contained also a CD4<sup>+</sup> T cell epitope in the peptide sequence (17,53) (Fig. 1B, IV). These two factors – the predominant local presentation of peptides and the provision of CD4<sup>+</sup> T-cell help (53,75)- which are lacking in most of the peptide vaccines used in human subjects, have greatly contributed to the enhanced efficacy of the early pre-clinical “short synthetic peptide” vaccines tested in rodents.

Second, the efficacy of many of the synthetic minimal CTL peptide vaccines that did seem to induce a proper CTL response, was assessed in prophylactic settings only (20,27,77,118). Importantly, the mice were lethally challenged when the CD8<sup>+</sup> T cell response was at its maximum and, therefore, able to rapidly deal with the infection/tumor challenge (20,27,77,118). In patients, peptide vaccinations are given in a situation of established diseases, requiring first and foremost a need to build up a highly effective Ag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell response, subsequently this immune response needs to be maintained for a long period of time to combat the disease. Notably, the early studies demonstrating that the use of minimal CTL peptide vaccines could induce tolerance when provided in oil-in-water formulations (IFA) (26,27,29,30) should have made us cautious in using minimal peptide-epitopes for vaccination. As we now know, the longer variants of these minimal CTL peptides provide a much safer form of vaccination (Chapter 2) (Fig. 1B. III). Moreover, since human beings display up to 8 different HLA class II molecules it is highly likely that these synthetic long CTL peptides (or proteins, Fig. 1A) contain CD4<sup>+</sup> T cell epitopes for one or more HLA class II molecules (62,65) (Fig. 1B, IV-V) and this will ensure full activation of professional APC allowing optimal priming of CD8<sup>+</sup> T cells (50,51). Covalent linking of synthetic TLR ligands to these synthetic peptide vaccines may be used to increase the magnitude of the CD8<sup>+</sup> T cell



response ((90,91,93) and Chapter 5) (Fig. 1C, III). Hence, for design of a successful peptide vaccine, it is important to adhere to a number of principles shown in Table 1.

**Table 1. Basic principles for peptide vaccination**

<b>Multiple epitopes</b>	Vaccine containing multiple epitopes induces a broad (CD4+ and CD8+) T cell repertoire that reduces the chance of immune escape variants	(62,65,103-105, 142-144)
<b>Broad applicability</b>	A (peptide) vaccine should be applicable for patients irrespective of their HLA type	(105)
<b>Ag targeting to professional APC</b>	Vaccines should contain peptides that predominantly target professional APC in order to prevent the possibility that T cells become tolerized by peptide presentation on non-professional APC	(75,81) and Chapter 3,5
<b>Extended duration of Ag presentation</b>	Peptide vaccines should install sustained duration of Ag presentation <i>in vivo</i> for optimal (CD8+) T cell clonal expansion to occur	(145-147) and Chapter 2,3
<b>Include CD4+ T cell help</b>	Concomitant induction of CD4+ and CD8+ T cell responses enhances and maintains strong CD8+ T cell effector responses	(21,23,28,52-57, 61-69,148-150) and Chapter 2 &4
<b>Include strong APC activating signals</b>	Provision of strong APC activating signals induces optimal activation and expansion of effector T cells	(49-51,74,75, 85-95,102) and Chapter 3,4,5
<b>Co-localization of Ag and APC activation</b>	Ag presentation and APC activating signals should be closely matched in time and location	(89-93) and Chapter 2,3,5

## FIVE-YEAR VIEW

In our opinion the use of synthetic vaccines comprising multiple CD4<sup>+</sup> and CD8<sup>+</sup> T-cell epitopes is only the start of the next generation of therapeutic vaccines (Fig. 1B, V). There is a need for additional regimens to further optimize the efficacy of the vaccine (Figure 2). A generic method to improve both the magnitude and the efficacy of the vaccine-induced T-cell response is to use longer peptides (75) (Fig. 1B, III-V). While most peptide vaccinations are already applied in oil-in-water adjuvants such as Montanide/ISA-51, occasionally supplemented with TLR-ligands such as MPL or CpG ((87,88) and reviewed in (31,85)), the inclusion of the immune potentiating TLR ligands will become general practice.



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Furthermore, vaccines exploiting antigens covalently linked to TLR ligands ((91,93,119) and Chapter 5) (Fig. 1C, III) increase the magnitude of the T-cell response as well as polarize the vaccine-induced immune response into a type 1 helper T cell/CTL response and are therefore likely to find their way into the clinic.

Vaccines are likely to be used in combination with the injection of T cell growth hormones such as IL-2 (43,120), IL-15 (121,122) or cytokines complexed to specific Abs or soluble receptors (123-125) resulting in enhanced *in vivo* efficacy, while greatly reducing the toxicity of these hormones (Figure 2, II). Pre-existing Ag-specific T regulatory T cells that can be boosted by the vaccine itself (126-128), should be removed first by for instance cyclophosphamide pre-treatment (129) or by the use of recombinant IL-2 linked to diphtheria toxin (DAB389IL-2) (130,131) (Figure 2, III). This will provide the right environment to further improve vaccine induced T cell response.

Notably, other emerging strategies to enhance this vaccine-induced T-cell response are injection of antibodies that either target specific members of the TNF receptor family, such as OX40 (132,133) and 4-1-BB (134) (Figure 2, I). Recently it was shown that successful peptide vaccines able to induce vast numbers of gp100-specific CD8<sup>+</sup> T-cells in melanoma patients, failed to induce concomitant clinical responses (135). This lack of clinical efficacy could be alleviated by blocking the inhibitory signal given to T cells using blocking antibodies against CTLA-4 (136,137) (Figure 2, I). Note, however, that the use of aCTLA-4 might, in the case that self-proteins are the target antigen, also result in the induction of auto-immunity (137). Alternative strategies can be the use of PD-L1 blocking Ab (reviewed in (138)). Additionally, inhibition of immune regulatory proteins in T cells - such as Cbl-b - could further improve the expansion and effectiveness of the T cell responses (Chapter 6).

In contrast to the treatment of hematological cancers, the homing of immune cells to solid tumors is essential but often fails to occur due to immunosuppressive mechanisms (139). Immunotherapy trials that combine vaccines together with modalities that are able to induce pro-inflammatory signals, either induced by chemotherapy (140), radiation, or by application of creams that contain TLR ligands on accessible lesions (141) are likely to be more successful in recruiting effector T cells (Figure 2, IV).

We therefore expect that the first successful product will be a completely synthetic inflammatory product comprising multiple Ag determinants - provided as long peptides or synthetic/recombinant proteins (Fig. 1A) - that will be ingested by professional APC and following Ag processing stimulates both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. This product that is likely to be given in combination with one or more therapeutic modalities that reduce immunosuppression and enhance T cell expansion as well as T cell homing.



## KEY ISSUES

- The lack of a profound insight in the mechanisms of peptide-induced CD8+ T-cell response by minimal CTL peptides, explains in part their failure to induce clinical responses in human vaccination trials.
- The induction of a strong T-cell response by (peptide) vaccines requires that both Ag presentation and the APC activating signals have to be matched both in time and in location.
- An inherent risk of (peptide) vaccines is that not only beneficial helper CD4+ T cells but also detrimental (pre-existing) regulatory T-cells are stimulated by vaccines.
- The immunotherapy of cancer will be a multi-modality approach in which synthetic (peptide) vaccines will be combined with strategies to decrease immune suppression, enhance T-cell expansion, effector potential, and T-cell homing.

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