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MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines

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Abstract: Major histocompatibility complex class I (MHC-I) presented peptide epitopes provide a 'window' into the changes occurring in a cell. Conventionally, these peptides are generated by proteolysis of endogenously synthesized proteins in the cytosol, loaded onto MHC-I molecules, and presented on the cell surface for surveillance by $CD8^+$ T cells. MHC-I restricted processing and presentation alerts the immune system to any infectious or tumorigenic processes unfolding intracellularly and provides potential targets for a cytotoxic T cell response. Therefore, therapeutic vaccines based on MHC-I presented peptide epitopes could, theoretically, induce $CD8^+$ T cell responses that have tangible clinical impacts on tumor eradication and patient survival. Three major methods have been used to identify MHC-I restricted epitopes for inclusion in peptide-based vaccines for cancer: genetic, motif prediction and, more recently, immunoproteomic analysis. Although the first two methods are capable of identifying T cell stimulatory epitopes, these have significant disadvantages and may not accurately represent epitopes presented by a tumor cell. In contrast, immunoproteomic methods can overcome these disadvantages and identify naturally processed and presented tumor associated epitopes that induce more clinically relevant tumor specific cytotoxic T cell responses. In this review, we discuss the importance of using the naturally presented MHC-I peptide repertoire in formulating peptide vaccines, the recent application of peptide-based vaccines in a variety of cancers, and highlight the pros and cons of the current state of peptide vaccines.

Keywords: cytotoxic T cells, epitopes, immunoproteomics, mass spectrometry, MHC class I, motif prediction, tumor-associated antigen, vaccine

Introduction

Major histocompatibility complex class I (MHC-I) molecules are present on the surface of all nucleated cells and display a large array of peptide epitopes for surveillance by the $CD8^+$ T cell repertoire. $CD8^+$ T cell responses are essential for the control and clearance of viral infections as well as for the elimination of transformed and tumorigenic cells. $CD8^+$ T cells effectively discriminate between healthy and infected or transformed cells via recognition of peptides associated with MHC-I (pMHC-I) molecules present on the cell surface. These peptides, which range from 8 to 11 amino acids in length, are typically derived from protein antigens in the cytosol that arise from conventional as well as cryptic translational reading frames [Shastri *et al.* 2002]. Classically, proteins synthesized in the cytosol undergo proteasomal degradation and the resulting peptides are transported into the endoplasmic reticulum (ER) and loaded onto MHC-I molecules [Blum *et al.* 2013]. Peptide loading results in stabilization of the class I molecules and transit to the cell surface where the complexes can be scanned by circulating $CD8^+$ T cells, a process called 'immune surveillance'. pMHC-I complexes are constantly shuttled to the cell surface; as such, the peptides bound to MHC-I serve as a readout of cellular events, including viral infection or tumorigenesis. This readout has considerable implications for the design and implementation of effective peptide-based cancer vaccines. In this review, we discuss the importance of using MHC-I presented peptide epitopes as a readout

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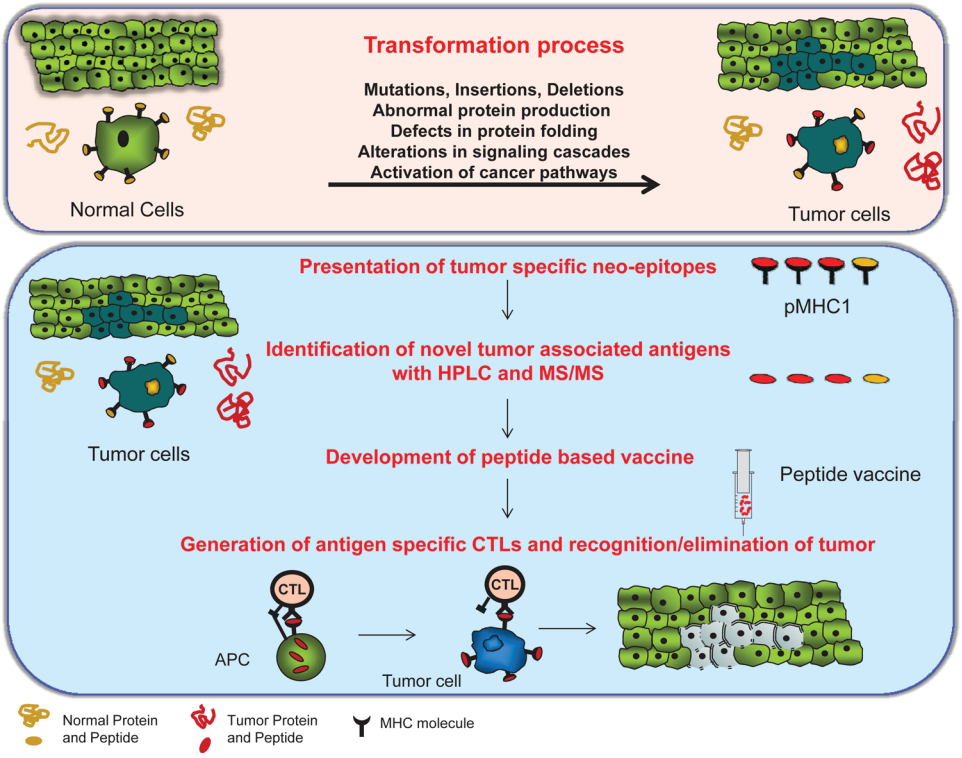

Figure 1. pMHC-1 (peptides associated with major histocompatibility complex class I) antigens for immunotherapy of cancer. Top: Transformation and subsequent tumorigenesis can be driven by multiple processes, generating abnormal proteins that are available for processing and presentation by the class I machinery. Bottom: The peptide epitopes generated by proteolysis of the abnormal proteins are attractive targets for peptide based vaccines as they represent the epitopes naturally presented by the tumor cell. These 'neo-epitopes' are isolated from tumor cells, identified by immunoproteomic methods, validated, and incorporated into a peptide-based vaccine to generate tumor-specific T cell responses.

of the internal proteome, or working state, of a cell. We review the recent literature on peptide-based therapeutic vaccines for human cancers highlighting the various delivery methods of these vaccines. Finally, we briefly discuss the pros and cons of pMHC-I based therapeutic vaccines and future directions in this field.

Importance of evaluating MHC-I presented peptide antigens for immunotherapy of cancer

Tumor development and maintenance of malignant phenotypes is driven by a wide range of abnormal cellular events including genetic mutations that result in changes in protein coding sequences, deletions, insertions, and the abnormal expression of critical genes involved in cancer transformation pathways [Hanahan and Weinberg, 2000]. Effective therapeutic cancer vaccines must take advantage of these genetic changes by selecting proteins involved in these cancer pathways in order to induce tumor-specific T cell responses (Figure 1). Identification of new tumor antigens, in general, is limited by certain aspects of the currently available technologies. For example, differential genomic and proteomic approaches identify over- and under-expressed proteins but are unable to identify very low abundant proteins that are often processed and presented by the MHC-I molecules as the true recognition targets for T cells. Indeed, the level of protein expression does not always correlate with MHC processing and presentation in cancer [Shastri *et al.* 2002]. Therefore, the most appropriate method for identifying truly relevant tumor-associated antigenic peptides is to analyze those actually presented by the MHC-I molecules on tumor cells. Described as 'nature's gene chip' by Shastri and colleagues [Shastri *et al.* 2002], the peptides displayed by MHC-I molecules represent the ever-changing proteome of the cell, in normal as well as in disease states, that could serve as targets for the $CD8^+$ T cell repertoire. In addition, the MHC-I

antigen presentation pathway incorporates cryptic antigenic peptides encoded by alternate reading frames generated by novel translational mechanisms [Starck and Shastri, 2011] and from prespliced mRNAs via a noncanonical translation mechanisms [Apcher *et al.* 2013], which makes the pMHC-I cellular state specific (i.e. tumor). Therefore, surveying peptides presented by the MHC-I molecules on the cell surface will reveal novel T cell targets for potential immune intervention as tumors have a distinct surface expression of peptides compared with their normal counterparts [Fortier *et al.* 2008]. Analysis of the peptide repertoire associated with the MHC-I molecules of cancer cells therefore provides a source for new tumor antigens for development of cancer immunotherapy (reviewed by Admon and colleagues [Admon *et al.* 2003]) and these antigens may serve as targets for the most difficult to treat tumors. Furthermore, the antigens identified by their MHC-I association on tumor cells should be tumor specific. Although normal tissues may express the antigen-coding genes, due to the differences in the regulation of expression and proteasomal processing, normal tissues in general do not present these antigenic epitopes in association with MHC-I molecules [Fortier *et al.* 2008]. Due to the lack of presentation of the epitopes in the context of MHC molecules in normal cells, the cytotoxic T lymphocytes (CTLs) do not recognize normal tissues, limiting the risk of autoimmunity [Hanada *et al.* 2004].

In the human immune system, MHC-I molecules are referred to as human leukocyte antigens (HLAs). Within the MHC, located on chromosome six, are three different genetic loci that encode MHC-I molecules; these molecules are referred to as HLA-A, HLA-B, and HLA-C. The genes encoded at each of these loci are extremely polymorphic, and thus, different individuals within the population express different class I MHC molecules on the surface of their cells. In addition, each MHC-I molecule has distinct peptide-binding capabilities determined, in part, by the amino acid composition that makes up the peptide-binding groove. Interestingly, peptides generated by the antigen processing machinery may bind to more than one HLA molecule. This property has allowed the categorization of MHC molecules into HLA supertypes, groups of HLA molecules that present at least one shared epitope. MHC-I associated peptides that have been found to bind to one member of the MHC allele supertype family (Al for example) are thought to be likely to bind to other members of the same supertype family (A32 for example) [Sidney *et al.* 2008]. As we will explain later, this could have considerable ramifications for peptide-based vaccination strategies.

The large number of pMHC-I complexes expressed at the cell surface combined with multiple pathways to generate epitopes provides a great resource for identifying physiologically and clinically relevant tumor-specific antigens (TSA) or tumor-associated antigens (TAAs). Undoubtedly, an examination of the peptides complexed with MHC-I molecules will reveal novel and highly immunogenic epitopes capable of inducing effective $CD8^+$ T cell responses. However, despite a growing body of literature indicating that $CD8^+$ T cells are naturally activated during an antitumor response [Traversari *et al.* 1992; Marincola *et al.* 1996; Nagorsen *et al.* 2000], these antitumor T cell responses often fail to eradicate tumors, in part due to suppression in the local tumor environment [Woo *et al.* 2001; Mougiakakos *et al.* 2010] and/or T cell induced exhaustion from continual antigen stimulation [Wherry, 2011; Baitsch *et al.* 2012]. Nevertheless, generating tumor-specific T cells capable of inducing tumor regression and/or elimination is a tangible possibility. Stimulating the expansion of new T cells through vaccination and/or reversing the exhaustion phenotypes of $CD8^+$ T cells are both attractive and feasible methods to generate robust antitumor responses [Parmiani *et al.* 2002; Baitsch *et al.* 2012; Sliwkowski and Mellman, 2013]. To this end, therapeutic vaccination has the capability to induce tumor-specific T cell responses to a number of TSAs and/or TAAs at once.

Current methods for identifying T cell epitopes for inclusion in peptide cancer vaccines

Currently, one of the major limitations in the development of cancer vaccines is the lack of clearly defined tumor antigens that are capable of being recognized by T cells. The definition of such antigens on tumors could provide the basis for a therapeutic vaccine, or for the stimulation of more effective CTLs for adoptive immunotherapy. One of the first methods used to identify tumor-specific peptides capable of binding to MHC molecules involved transfecting cDNA generated from tumor cells into recipient antigen-presenting cells. In this genetic approach, the proteins expressed

from cDNA transfection would be translated and processed into epitopes that could load onto MHC-I molecules. Using this technique, an HLA-A1 restricted epitope from MAGE-1 [Van Der Bruggen *et al.* 1991], an HLA-A2 restricted epitope from tyrosinase [Brichard *et al.* 1993], and an HLA-A2 restricted epitope from MART-1 [Kawakami *et al.* 1994] were identified and capable of inducing robust $CD8^+$ T cell responses in melanoma. This approach was, and to some extent still is, an attractive technique because cDNA can be transfected into cells expressing different MHC molecules allowing for a more broad characterization of tumor-specific peptides. However, genetic approaches to identifying tumor-specific epitopes are not without drawbacks. First, any differences in protein expression between the cDNA transfected cell and the tumor cell from which the cDNA was derived may alter the balance of antigen processing and presentation. This may generate more pMHC-I complexes on the transfected cell than on the natural tumor cell and, potentially, more robust T cell responses. In these cases, the stimulatory impact of these epitopes would be overestimated. Differences in the ability of antigen presenting cells to post-translationally modify proteins may also impact epitope discovery. Skipper and colleagues demonstrated that an epitope generated from tyrosinase is modified, changing an asparagine to aspartic acid which generates a more robust $CD8^+$ T cell response, despite no differences in peptide binding to HLA-A2 [Skipper *et al.* 1996]. Finally, and perhaps more importantly, cDNA expression in different cell types may not generate physiologically relevant epitopes. It is known that different cell types have different levels of proteolytic activity [Delamarre *et al.* 2005; Savina *et al.* 2006] and therefore, epitopes generated in the antigen-presenting cells expressing the cDNA may not be the same as those generated in the tumor cell itself.

A second method to identify potential MHC-I binding peptides from already known tumor antigens (identified by differential genomic and proteomic methods) is motif prediction: using pMHC-I binding algorithms that estimate how well peptides will bind to a specific MHC-I molecule [Schultze and Vonderheide, 2001; Shastri *et al.* 2002; Admon *et al.* 2003]. These algorithms are based on patterns obtained from peptides known to bind MHC-I molecules with scores being assessed by evaluating specific anchor residues between the peptide and MHC binding groove. Predictions can be further honed by examining potential proteasomal cleavage events in the parent protein [Nussbaum *et al.* 2001; Stevanovic, 2002], thus creating an 'optimal' epitope. Using an epitope prediction technique, Fisk and colleagues identified 19 peptides within the Her-2 protein sequence that were predicted to bind to HLA-A2 molecules [Fisk *et al.* 1995]. Interestingly, only one peptide was able to induce tumor-specific $CD8^+$ mediated lysis for all CTL lines tested. Similarly, epitope binding predictions led to the identification of an HLA-A2 restricted epitope from MUC-1 that is presented on a variety of tumors [Brossart *et al.* 1999] and HLA-A3 restricted epitopes derived from carcinoembryonic antigen (CEA) and Her2/neu [Kawashima *et al.* 1999]. In fact, the epitopes identified from CEA and Her-2/neu bind to multiple HLA alleles in the A3 superfamily, suggesting that these peptides could overcome some differences in HLA expression in patient-to-patient comparisons [Kawashima *et al.* 1999]. Since these initial studies, peptide-binding algorithms have been used in an attempt to predict epitopes from virtually all known tumor antigens, including p53 [Papadopoulos *et al.* 1999], MAGE [Akiyama *et al.* 2012], HCA587 [Li *et al.* 2005], TRAG-3 [Zhu *et al.* 2003], and ALK [Ait-Tahar *et al.* 2006]. However, like the genetic approach to identifying epitopes, peptide prediction algorithms are not reliable. One major reason is that prediction algorithms do not accurately represent what occurs in an antigen presenting cell. Predicted 'binders' to MHC-I may not be generated due to proteolytic events or may not efficiently stimulate $CD8^+$ T cells [Fisk *et al.* 1995] (also found in the present authors' unpublished observations). Similarly, those peptides not predicted to bind to a specific HLA molecule with high affinity may in fact induce productive T cell responses. In addition, motif prediction methods may be limited in identifying subdominant epitopes which are likely to escape tolerance mechanism [Thomas *et al.* 2007]. Comparison of the motif prediction method with direct mass spectrometry analysis of endogenously presented epitopes isolated from virus-infected cells revealed a high number of predicted epitopes were not processed and presented by the infected cells [Zhong *et al.* 2003]. These findings indicate that the complexity of the motif predicted epitopes combined with $CD8^+$ T cell-based screening of functional epitopes may miss hidden subdominant epitopes.

In the last decade, direct identification of MHC-I presented epitopes from tumors or infected cells

has emerged as an alternate to the motif prediction method, a process termed immunoproteomics [Purcell and Gorman, 2004]. The analytical challenge lies in the discrimination between the tumor-related peptides among a majority of nondisease-related peptides that are presented on the cell surface [De Jong, 1998]. This could be overcome by cancer specific database search of the identified peptides to select those that are derived from tumorigenesis pathway involved proteins [Hanahan and Weinberg, 2000]. Immunoproteomic analysis is generally based on the isolation of the MHC-peptide complexes from tumor cells and elution of the bound peptides from the MHC molecules followed by offline high-performance liquid chromatography (HPLC) fractionation [Rotzschke *et al.* 1990; Falk *et al.* 1991] and online HPLC fractionation combined with mass spectrometry [Hunt *et al.* 1992; Di Marzo Veronese *et al.* 1996; Van Els *et al.* 2000; Berzofsky *et al.* 2001; Hickman *et al.* 2003; Lemmel *et al.* 2004]. The peptides are then validated by both *in vitro* and *in vivo* assays. Elution of peptides from both mouse and human MHC-I molecules identified MHC-I restricted epitopes from tumor (i.e. P815 and JY cells) [Falk *et al.* 1991] and influenza-infected cells [Rotzschke *et al.* 1990] and nine HLA-A2 restricted epitopes from the human B-cell lymphoblastoid line C1R.A21 [Hunt *et al.* 1992]. Since these pioneering studies, our group [Shetty *et al.* 2011, 2012; Testa *et al.* 2012a, 2012b] and others [Skipper *et al.* 1999; Hogan *et al.* 2003, 2004; Zarling *et al.* 2006; Hawkins *et al.* 2008; Feyerabend *et al.* 2009; Haen and Rammensee, 2013] have applied this technique to identify naturally processed epitopes from various tumor or infected cells capable of inducing $CD8^+$ T cell responses. This immunoproteomic approach to epitope identification has significant advantages. First and foremost, naturally processed epitopes present on the surface of tumor cells represent the most clinically relevant targets for vaccination or immunotherapy design. Differences in protein expression levels and antigen processing are minimized greatly in comparison with other identification techniques. Second, the same tumor cell sample can be used to identify epitopes that will bind to multiple MHC-I alleles, either via superfamily mapping or the use of allele specific antibodies during the discovery process. Importantly however, after identification of the epitope, validation must ensure that the epitopes are not present on normal tissues either by similar immunoproteomic analysis or cellular assays demonstrating no $CD8^+$ T cell reactivity to normal cells.

Peptide-based vaccines in the clinical setting

Peptide-based vaccines have enjoyed minimal success thus far in the clinical setting. In this section, we will review recent developments in peptide-based cancer vaccines for a select number of malignancies focusing on peptide composition and the antitumor immune response generated. To date, most of the peptide-based vaccines tested in the late stage clinical studies include peptides identified by motif prediction methodology with fewer exceptions mainly in melanoma and renal carcinoma.

Melanoma

The vast majority of research into peptide-based therapeutic vaccines has centered on melanoma, as there are many well described MHC-I restricted epitopes available for testing. An epitope derived from the MAGE-1 protein was the first to be tested in a peptide-based clinical trial. Although epitope specific $CD8^+$ T cells could be generated and expanded *in vitro* post-vaccination, no clinical responses in patients were observed [Hu *et al.* 1996]. Despite these results, this study was important as it reinforced the idea that $CD8^+$ T cells could be induced to generate an antitumor response. More recent studies of peptide-based (most of them identified by immunoproteomic methods) vaccines have utilized a multi-epitope approach in order to induce a broader range of T cell specificities and potentially overcome the problem of antigen loss variants that arise during cancer progression [Admon *et al.* 2003; Slingluff, 2011]. In a randomized phase II clinical trial of patients with stage IIB to IV melanoma, Slingluff and colleagues compared the effectiveness of a 12 *versus* a 4 MHC-I peptide-based vaccine [Slingluff *et al.* 2007]. Vaccines contained tetanus helper peptide, granulocyte-macrophage colony-stimulating factor (GM-CSF), and Montanide ISV-51 and were given intradermal (i.d.) and subcutaneous (s.c.) $CD8^+$ T cell responses induced after vaccination with the 12 peptide vaccine were more broad and robust as characterized by $CD8^+$ interferon (IFN)-γ secretion, however no clinical efficacy was observed in either vaccine [Slingluff *et al.* 2007]. Importantly however, the data demonstrated that multiple peptides could be injected safely and at the same site with no effect on competition for class I binding. In contrast, clinical efficacy was observed in a trial of a three peptide vaccine given s.q. with Montanide ISV-51 and containing GM-CSF, IFNα2b, or both [Kirkwood

et al. 2009]. Data from 115 patients with stage IV melanoma were analyzed and demonstrated that functional responses to the peptides (as judged by IFN-γ secretion) were correlated with a roughly 8-month increase in overall survival with two complete remission and six partial remission cases, with no differences observed between any of the cytokine groups [Kirkwood *et al.* 2009]. The inclusion of cytokines in vaccines needs to be explored further in order to enhance the antitumor effectiveness of the $CD8^+$ T cells. Indeed much of the data to date indicates that certain cytokines, at least at the doses used currently, are not effective at enhancing antitumor responses and in fact decrease $CD4^+$ and $CD8^+$ T cell responses [Slingluff *et al.* 2009] and may induce the accumulation of regulatory T cells (T_{REGs}) [Block *et al.* 2011].

Because $CD4^+$ T cell responses can potentiate $CD8^+$ T cell responses, multiepitope vaccines may also need to include $CD4^+$ activating peptides. Slingluff and colleagues monitored $CD4^+$ and $CD8^+$ T cell responses in 175 patients with stage IV melanoma after administration of a 12 peptide vaccine alone, with tetanus peptide, with 6 confirmed melanoma helper epitopes, or a vaccine of the 6 melanoma helper epitopes alone [Slingluff *et al.* 2013]. Vaccines were administered i.d. and s.q. emulsified in Montanide ISV-51. Although including tetanus helper peptide in vaccines enhanced $CD8^+$ T cell responses, it did not have any impact on overall survival. In direct contrast, including melanoma-specific helper peptides did not enhance $CD8^+$ T cell responses but was associated with increase in survival [Slingluff *et al.* 2013]. This data suggest that absolute numbers of $CD8^+$ T cells might not be the most appropriate way of assessing vaccine-induced responses and that there exists an optimal ratio between the $CD8^+$ and $CD4^+$ compartment for effective antitumor responses. Nevertheless, continuous exploration of vaccine strategies to incorporate class II epitopes is of high priority.

Dendritic cells (DCs) are considered one of the most important cells in initiating an immune response and as such have received much attention in designing peptide-based vaccines for cancers. Lesterhuis and colleagues evaluated the ability of a peptide pulsed DC vaccine to induce clinical responses in metastatic melanoma patients [Lesterhuis *et al.* 2011]. DCs were generated from peripheral blood mononuclear cells (PBMCs) and pulsed with tyrosinase and wildtype gp100 peptides or modified versions with higher binding affinity to HLA-A2 and injected intravenously (i.v.) and i.d. into patients. Although clinical responses were limited, 2 out of 27 patients had responses lasting at least 8 months [Lesterhuis *et al.* 2011]. Oshita and colleagues evaluated DC induced clinical responses in a phase II trial of metastatic melanoma patients. Melanoma-specific HLA-A2 and HLA-A24 peptides were loaded onto DCs generated from patient blood and administered subcutaneously (s.c.) over a period of 5 months [Oshita *et al.* 2012]. A total of 18 (out of 24; 75%) patients mounted specific $CD8^+$ T cell responses as assessed by IFN-γ ELISpot and the majority of these patients had T_H1 type cytokine skewing. Despite most patients progressing clinically, six patients experienced stable disease and one patient experienced a partial response.

Colon cancer

In contrast to melanoma vaccines, peptide vaccines for colorectal cancer have typically relied on a single peptide injected with adjuvant, usually Montanide ISA-51. In 2004, an HLA-A24 restricted $CD8^+$ T cell epitope from the survivin protein, called survivin-2B80-88, was injected s.q. into patients with colon cancer [Tsuruma *et al.* 2004]. No adjuvant appeared to be used so it is not surprising that no clinical response were observed except for a minor increase in survivin tetramer positive $CD8^+$ T cells in a handful of patients. Building off of this study, the group then combined survivin peptide with Montanide ISA-51 with or without IFN-α in patients with unresectable colon cancer [Kameshima *et al.* 2011]. Of the five patients that received only peptide and Montanide, one had stable disease. In contrast, four out of the eight patients receiving peptide and Montanide with IFN-α had stable disease that was accompanied by decreased levels of the colon cancer tumor marker CEA [Kameshima *et al.* 2011]. Other peptide-based vaccines have been tested clinically but these do not induce $CD8^+$ T cell responses. Notably, vaccination of patients with an extended p53 peptide induced sustained $CD4^+$ T cell responses [Speetjens *et al.* 2009] that were enhanced (i.e. higher levels of IFN-γ) when administered with IFN-α [Zeestraten *et al.* 2013].

DC-based vaccines for colon cancer have also been tested in the clinical setting. In a phase I/II clinical trial, Kavanagh and colleagues evaluated the ability of matured DCs to activate $CD8^+$ T

cells in colon cancer patients [Kavanagh *et al.* 2007]. DCs were pulsed with peptides derived from CEA, Her2-neu, MAGE-2, and MAGE-3 and injected over a period of 3 weeks. Only 3 out of 21 patients made specific $CD8^+$ T cell responses that were directed at a single CEA epitope, though expansion of other peptide specific T cells was observed after *in vitro* T cell stimulation [Kavanagh *et al.* 2007]. Despite the ability to induce T cell responses, no significant clinical benefits were observed. Lesterhuis and colleagues also evaluated DCs as a vaccine candidate comparing peptide pulsing with mRNA electroporation [Lesterhuis *et al.* 2010]. DCs were pulsed with the CEA peptide CAP-1 or electroporated with CEA mRNA and delivered i.d. and i.v. a total of three times. A total of 8 out of 11 patients receiving peptide pulsed DCs mounted a $CD8^+$ T cell response detectable by tetramer staining compared with 2 out of 5 patients in the electroporated group [Lesterhuis *et al.* 2010]. This latter study reinforces the need to identify naturally processed epitopes presented on tumor cells as it is not clear that the electroporated cells generated the CAP-1 epitope efficiently.

Breast cancer

Tsuruma and colleagues tested a survivin peptide vaccine with or without Montanide ISA-51 in a phase I trial of patients with breast cancer [Tsuruma *et al.* 2008]. As in previous studies, no clinical responses were observed, but the four patients receiving the peptide with Montanide vaccine had more survivin tetramer positive $CD8^+$ T cells with one patient making a specific, IFN-γ functional response [Tsuruma *et al.* 2008]. A more common target of breast cancer peptide vaccines is the Her2-neu antigen. Two recent phase I or phase II clinical trials evaluated immune responses after vaccination of the E75 or GP2 peptide vaccine in HLA-A2 expressing patients with disease-free breast cancer. Together, the studies indicated that both the E75 and GP2 epitopes were immunogenic, induced epitope specific $CD8^+$ T cells [Carmichael *et al.* 2010; Mittendorf *et al.* 2012] and, in a subset of patients, potentially prolong disease-free survival states [Mittendorf *et al.* 2012]. Multi-epitope breast cancer vaccines have also been tested in clinical trials. A mixture of 12 HLA-A2 restricted epitopes identified by the immunoproteomic method in ovarian cancers [Ramakrishna *et al.* 2003] was combined with Montanide ISA-51 and GM-CSF and delivered s.q. and i.d. into patients with resected breast cancer [Morse *et al.* 2011]. Patients that received a high-dose vaccine made broader $CD8^+$ T cell responses than patients that received a low-dose vaccine (as assessed by IFN-γ secretion in an ELISpot assay; >9 responses in high dose, 0–4 response in low dose) suggesting that a multi-epitope vaccine can induce specific T cell responses, but that the effectiveness of these may depend on dose of peptide given.

Generating Her2-neu specific T cell responses in breast cancer is also possible via DC-based vaccines. Patients with confirmed ductal carcinoma *in situ* (DCIS) were injected with DCs pulsed with a group of Her-2/neu peptides (six MHC class II peptides and two MHC class I restricted peptides) [Sharma *et al.* 2012]. A total of 85% of patients enrolled had detectable $CD4^+$ and $CD8^+$ T cell responses to the vaccine, and it seems likely that these responses led to a decrease in Her2-neu expression in these patients [Sharma *et al.* 2012] although a decrease in antigen expression is not necessarily indicative of complete elimination of the cancer. In a second study of patients with DCIS, DCs were pulsed with a mixture of class I and II binding peptides, matured *in vitro* with IFN-γ and lipopolysaccharide (LPS), and injected into the patient [Koski *et al.* 2012]. This immunization strategy resulted in functional (IFNγ secreting) $CD8^+$ T cells in 11/13 patients expressing the HLA-A2 allele and functional $CD4^+$ T cell responses in 22/25 patients enrolled in the study.

Renal cancer

Renal cell carcinoma (RCC) is one of the most common types of cancers that occur in the adult population with metastatic RCC having a 5-year survival rate of less than 10% [Schrader *et al.* 2006]. Vascular endothelial growth factor receptor 1 (VEGFR1) plays a key role in the progression of RCC and therefore peptides derived from this protein could serve as an attractive target for T cell based therapies. To this end, Yoshimura and colleagues investigated the effectiveness of a two-peptide VEGFR1 vaccine (one HLA-A2 and one HLA-A24 restricted peptide) delivered s.q. in Montanide ISA-51 [Yoshimura *et al.* 2013]. A total of 15 out of 18 patients had specific $CD8^+$ T cell responses, complete with IFN-γ secretion. Clinically, two patients had a partial response and nine patients had stable disease for at least 5 months [Yoshimura *et al.* 2013]. Using the immunoproteomic approach, Walter and colleagues

identified nine HLA-A2 restricted epitopes from RCC patient samples [Walter *et al.* 2012]. These epitopes were incorporated into a vaccine called IMA901 that was synthesized and injected i.d. along with GM-CSF into patients with RCC. $CD8^+$ T cell responses to multiple antigens were associated with control of the disease. Further, inclusion of cyclophosphamide 3 days before IMA901 injection prolonged survival and reduced the number of regulatory T cells [Walter *et al.* 2012]. This latter point is critical: because T_{REGs} are well represented in the tumor microenvironment, peptide-based vaccines may need a T_{REG} depleting step prior to injection or other modulation of the anti-inflammatory environment by concomitant cytokine treatment. However, not all cytokines are ideal in this application. In trials of DC-based vaccines combined with interleukin (IL)-2 administration, T_{REGs} were induced to significantly higher levels than before treatment, albeit transiently [Lemoine *et al.* 2009; Berntsen *et al.* 2010].

Other malignancies

Peptide-based vaccines have also been evaluated in many other clinical settings. In a phase 1 clinical trial, 15 HLA-A2+ patients with stage III–IV non-small cell lung cancer were vaccinated with a peptide vaccine derived from indoleamine 2,3 dioxygenase (IDO) [Zeeberg Iversen *et al.* 2013]. A total of 6 out of 15 of the patients had stable disease and overall survival was increased ~18 months compared with HLA-A2-negative patients who were unvaccinated. Sawada and colleagues demonstrated that vaccination of patients with hepatocellular carcinoma using a peptide derived from glypican-3 resulted in $CD8^+$ T cell expansion with an improvement in overall survival in patients with robust GPC3 responses [Sawada *et al.* 2012]. In phase I clinical studies, a multi-epitope-based vaccine demonstrated CD8+ T cell responses and delay in progression of disease in ovarian and breast [Morse *et al.* 2011] and prostate cancer [Berinstein *et al.* 2012]. Finally, a multi-epitope vaccination approach was used in a phase I trial of patients with biliary tract cancer and resulted in a detectable clinical response in six of the nine patients [Aruga *et al.* 2013].

Advantages and disadvantages of peptide vaccines: where do we go from here?

Overall, the data discussed above indicate that peptide vaccines are capable of inducing robust $CD8^+$ T cell responses that, in some cases, provide clinical benefit to patients. Peptide based vaccines have significant advantages as a cancer immunotherapy option. First, these vaccines are flexible in their design and can accommodate many peptide epitopes in a single dose. This allows for multiple MHC-I epitopes to be included to initiate a T cell response. This is an important feature because not all individuals share the same MHC alleles; peptides that bind to single alleles (i.e. HLA-A2 *or* HLA-A24) and peptides that bind to multiple alleles (i.e. HLA-A2 *and* HLA-A24) can be included in the same formulation. Thus, a vaccine derived from naturally processed peptides can be given to individuals with a wide diversity in their MHC alleles and still be effective. Second, a multi-epitope vaccine may protect against tumor resistance due to antigen downregulation by inducing a more broad, oligoclonal response. Although multiple epitopes from a single antigen have been identified and might overcome HLA restriction (i.e. MAGE-n [Zhang *et al.* 2010], survivin [Tsuruma *et al.* 2008; Shen *et al.* 2013], and CEA [Nukaya *et al.* 1999; Keogh *et al.* 2001]), it is important that the epitopes included in such a vaccine be derived from different parent proteins. This not only will increase the clonality of the T cell response but also prevent tumor cells from downregulating a single protein and escaping the T cell response induced by the vaccine. Finally, peptide-based vaccines can also incorporate MHC class II restricted epitopes to activate $CD4^+$ T cells and/or B cell epitopes to activate T helper and antibody-mediated responses. Together, a complete adaptive immune response could prove to be a more effective and robust way by which to eliminate tumors. While a protein-based vaccine might be attractive for similar reasons, antigen processing can be markedly different from cell to cell. Downregulation of proteasomal subunits, including the IFN-γ inducible immunoproteasome, occurs in numerous cancers, such as B cell lymphoma and breast cancer [Seliger *et al.* 2000]. This downregulation alters the cleavage specificities of the tumor proteasome; therefore, epitopes generated in antigen presenting cells that process the protein vaccine via a 'normal' proteasome may not accurately reflect epitopes generated by the class I machinery of tumor cells thereby limiting the effectiveness of the $CD8^+$ T cell response. Despite these advantages, peptide-based vaccine strategies are not without their downfalls. First and foremost, in order for the vaccine to be effective the tumors must be expressing the antigens included in the vaccine formulation. Ideally, the

tumors should be *presenting* the epitopes included in the vaccine, which is a major reason for using an immunoproteomic approach for the discovery and selection of antigens in vaccine development. Second, peptide-based vaccination has been shown to induce the accumulation of immunosuppressive regulatory T cells [Lemoine *et al.* 2009; Berntsen *et al.* 2010; Block *et al.* 2011] which would limit vaccine utility *in vivo.* Finally, in some instances peptide vaccines may not be enough to eradicate tumors from patients, depending on staging of the disease. Importantly, potential solutions exist to prevent or mitigate each of these limitations.

In addition to identifying novel peptides, there are several avenues of research needed to improve the effectiveness of peptide vaccines. First, it is possible that improvements in adjuvant technology will enhance the T cell responses generated during vaccination. One active area of research in this regard is including TLR agonists in vaccine formulations, as these have been shown to heighten protective immune responses [Mahla *et al.* 2013]. Second, inclusion of cytokines in the vaccine formulation to enhance the immune responses may also improve vaccine effectiveness. As described above, cytokines included in some formulations induced the formation of T_{REGs} [Lemoine *et al.* 2009; Berntsen *et al.* 2010; Block *et al.* 2011]. It will be critical to understand the appropriate cytokines or adjuvants in the form of antigen delivery (i.e. viral or bacterial vector or biodegradable nanoparticle based) to include that will enhance responses without inducing an immunosuppressive environment. Along these lines, and perhaps most critical to inducing effective response after vaccination, is determining how to limit the formation of T_{REG_S} either by including a cytokine or adjuvant in the vaccine or via pretreatment with certain drugs as demonstrated by Walter and colleagues [Walter *et al.* 2012].

Peptide-based vaccines, despite their limited effectiveness to date, have shown promise and progress in the clinic. Identifying novel and perhaps more immunogenic peptides through an immunoproteomics approach combined with a better understanding of adjuvant and cytokine therapy should result in more clinically effective vaccine regimens.

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Conflict of interest statement

None of the authors have relevant financial interests related to this manuscript.

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