Design of Peptide-Based Vaccines for Cancer

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Abstract: The immune system responds efficiently to bacteria, viruses and other agents however, the immune response to cancers is not as effective. In most cases other than specific genetic rearrangements leading to non-self proteins such as in leukemia and idiotypes in lymphoma, tumor associated proteins are self proteins and are not recognized by the immune system to prevent malignancy. In most cancers, patients develop antibodies and/or CTL-precursors to tumor associated antigens but are not effective in generating a therapeutic immune response. Adjuvants have been used with either whole tumors, subunits or peptides with the aim of increasing their immunity. Whole tumor antigens have certain advantages associated with it, such as ready availability as recombinant proteins, potential epitopes that can be presented by a number of MHC class I/II alleles and antibody development. The methods of identification of CD8 and CD4 epitopes either by use of epitope prediction algorithms or use of transgenic mice has made the use of defined synthetic peptides more attractive. The possibility to synthesize long peptides and introduce multiple epitopes (CD4 or CD8) from single or multiple antigens makes peptide a viable alternative to whole proteins. As an alternative to totally synthetic peptide constructs or polymers, polytopes have been generated by genetic engineering methods. In addition, to deliver immunogens to and to activate DC, receptor-mediated delivery of peptides using antibodies, cytokines and carbohydrates have been used. This review will encompass the various strategies, preclinical and clinical applications in designing peptide-based vaccines for cancer.

Keywords: Peptide, vaccine, mimotope, MAP, cancer, multiepitope, tumor associated antigens.

INTRODUCTION

The first vaccine was developed in 1796 when Dr Edward Jenner performed the first vaccination with infectious fluid from the hands of milkmaids infected with cowpox into the arm of a healthy boy. The boy showed symptoms of cowpox infection, then recovered and was later infected with smallpox but did not show any clinical signs of smallpox. This principle of using a less harmful infectious organism to cross protect against a harmful infectious agent spurred the field of vaccination and resulted in highly effective inactivated

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(flu, cholera, plague, and hepatitis A), live/attenuated (flu, yellow fever, measles, rubella) or toxoid (tetanus and diphtheria) vaccines against infectious diseases. Vaccines have contributed to the eradication of smallpox - the last case in Ethiopia in 1976 and in 1980, the WHO announced that vaccines had been successful at eradicating smallpox from the world, one of the most contagious and deadly diseases known to man. Incidence of other diseases such as rubella, polio, measles, mumps, chickenpox, and typhoid has also decreased significantly over the past 100 years.

The use of immunotherapy to combat cancer originated from the initial attempts of Dr William Coley in the late 19^{th} century who used bacterial extracts (Coley's toxin) to treat patients with advanced cancer [1, 2] Coley's toxin was remarkably effective with a strong febrile response crucial for regression. A retrospective study compared the 10 year survival rates of patients treated with Coley's toxin with those treated with modern treatments and concluded that despite the vast amounts of money spent on developing new therapies, patients receiving modern treatments did not fare any better than those receiving Coley's toxin more than 100 years ago. Interestingly BCG is the only bacterial vaccine in use and the only effective treatment for superficial bladder cancer when given intravesically.

Despite all the efforts on research and clinical trials there is still no effective cancer vaccine. The discovery that tumors express antigens that can be targeted by cytotoxic T lymphocytes (CTL) is the fundamental concept behind developing current immunotherapy [3]. The generation of a CD8 T cell response is necessary for the eradication of tumors. However, to expand and sustain the CTL response, CD4+ T cell help is crucial. There is a large number of tumor associated antigens (TAA) identified and used as targets for immunotherapy. These belong to several classes a) those not expressed by normal cells such as viral antigens [human papillomavirus-16 (E6, E7). HBV (HbsAg, HBVPresS2, HbcAg), EBV (gp340/320, EBNA-3A)], idiotypes of B cell lymphomas b) products of rearranged genes (Bcr-Abl, $EGFR_{vIII}$) or mutated genes (p53, ras p21), c) cancer-testis antigens (MAGE, NY-ESO) or d) antigens with low expression on normal cells and elevated expression on tumor cells (MUC1, carcinoembryonic antigen, HER-2/*neu*, p53). Various approaches such as whole tumor cells, recombinant proteins, peptides and delivery systems such as antigen presenting cell (APC) targeting, microparticulate sytems, adjuvants, pulsed dendritic cells (DC), DNA, RNA and recombinant viruses have all been used for vaccination.

Immunization with whole proteins have some advantages, as they potentially have multiple CTL epitopes and T helper cell epitopes not restricted to one HLA type. However, since most TAAs are self antigens T cells that recognize the high affinity immunodominant epitopes may have been deleted during lymphocyte development. Rationally designed peptide vaccines may be able to overcome some of the limitations of other modalities of vaccination. Multiepitope vaccines incorporating, multiple CTL epitopes encompassing HLA-A2, A3, A24 and promiscous T helper epitopes from one or more antigens together with an appropriate adjuvant or danger signal are prerequisites for better cancer vaccines. Using information from MHC/peptide crystal structures low affinity subdominant CTL epitopes maybe also be optimized.

In this review we will discuss novel TAAs and the design of synthetic peptide vaccines with particular reference to the most recent literature. Approaches that have been used to deliver multiepitope vaccines and the use of B/T cell mimotopes to overcome peripheral tolerance to TAA will also be discussed.

IDENTIFICATION OF MHC CLASS I AND CLASS II EPITOPES

The specificity of MHC class I molecules has been determined using pool sequencing of eluted peptides, binding studies and/or phage display library analysis [4]. These results indicated the presence of conserved/consensus anchor residues amongst the high affinity binding peptides. In the process however low-to-medium affinity peptides or medium-high affinity non-canonical peptides were missed in this analysis. As a result the identification of such non-canonical peptides were not predictable from the protein primary sequence. Given that it is impractical to test all possible overlapping peptides of proteins, numerous programs have been developed to predict peptides with the potential to bind to MHC molecules based on the canonical anchor motifs known for high affinity peptide binding [4-5]. Systematic screening from TAA for potential MHC class I epitopes, using the programs (SYFPEITHI [5]; MHCPEP [6]; HLA-BIND [7]) is a relatively simple process. However, most of these peptides identified by these programs do not induce T cell responses. Furthermore, non-canonical binding peptides are not predicted / identified using these programs. New methods / programs are emerging which are able to predict an array of peptides which can bind non-conventionally to MHC class I molecules. EpiMatrix and Conservatrix search for unique or multi-HLA restricted (promiscuous) T cell epitopes and identifies epitopes that are conserved across variant strains of the same pathogen [8]. Quantitative structure-activity relationship (QSAR) methods have been used to identify peptides which bind to more than one allele [9, 10]. A new method was developed for predicting MHC binding of peptides based on peptide property models constructed using biophysical parameters of the constituent amino acids and a training set of known binders [11]. This method has been constructed based on the tumor associated antigens, MART-1, S-100, MBP, CD63, MUC1, p53, cyclin B1, HER-2/neu and CEA and a number of low-medium affinity peptides were identified which were usually missed in other standard prediction programs. In addition to using standard programs to predict binding of peptides to MHC class I, programs have been developed which determine which peptide sequences are cleaved in the proteosome, are transported through TAP and bind to MHC class I [12]. Likewise, Immune Epitope Database (IEDB) (web: http://tools.immuneepitope.org/main/index.html) provides a collection of tools for the prediction and analysis of immune epitopes (T and B cell epitope prediction). The T cell epitope prediction tools includes the prediction of peptides binding to MHC class I and MHC class II molecules based on their IC_{50} values. In addition, a tool is available which predicts epitopes based upon proteasomal processing, TAP transport and MHC binding to produce peptides intrinsic potential of being a T cell epitope. IEDB also includes NetChop, a predictor of proteasomal processing and NetCTL [13] which predicts CTL epitopes in a protein sequence (web server: http://tools.immuneepitope.org/main/index.html). Thus, the peptides identified would most likely induce CTL responses compared to peptides that have been identified to only bind to MHC class I. Furthermore, the method PepScope has been established and identifies many potential T cell epitopes based on new anchor motifs which would normally be missed with current prediction programs [14] In addition to MHC class I prediction programs, there are numerous methods which identify MHC class II binding peptides. RANKPEP [15], Gibbs motif sampler [16] and TEPITOPE [17] are commonly used. More recently, a program was developed based on the average relative binding matrix method that predicts IC_{50} values of different length peptides and to bind to MHC class II molecules (web server: http://epitope.liai.org:8080/matrix) [18]. Furthermore, MULTIPRED was designed to predict peptides binding to multiple MHC class I and II alleles. This method also allows the prediction of peptides that promiscuously bind to multiple HLA alleles within one supertype using hidden Markov models and artificial neural network methods (http://antigen.i2r.a-star.edu.sg/multipred/) [19]. SMM-align [20], was developed to

identify a preference for hydrophobic or neutral amino acids at the anchors which is more superior to TEPITOPE which favors basic amino acids at most anchor positions. Much work is required in (i) identifying non-canonical peptides from proteins and (ii) accurately identifying peptides from prediction programs which bind to MHC class I/II and induce immune responses.

STRUCTURE BASED MODIFICATIONS OF CLASS I AND CLASS II EPITOPES

Peptide epitope modifications can enhance peptide immunogenicity by improving binding and stability. Tolerance can be overcome by selecting low affinity peptides and modifying the 'anchor' residues to increase binding affinity. Alternatively, the TCR contact residues can be modified to improve T cell activation, Fig. (**1**).

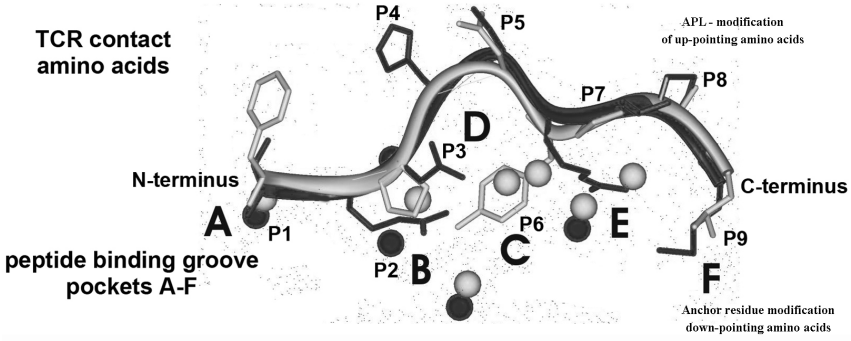

Fig. (1). Side view of peptides bound to MHC class I, H-2K^b. Peptide side chains which point 'down', bind into pockets (A-F), peptide side chains which point 'up', interact with the T cell receptor (TCR). Modifications can be made to the 'up-pointing' amino acids or to the 'down-pointing' amino acids.

'Anchor' Residue Modification

Tumor antigens are self antigens, and therefore tolerance is one of the limiting factors. Self antigens are poorly immunogenic for CTL. High vaccination efficiency has been demonstrated for low-affinity epitopes, derived from the murine telomerase reverse transcriptase. Anchor modifications (Fig. (**1**)) improved MHC binding affinity and exhibited potent anti-tumor immunity with no autoimmune responses in mice [21]. Enhanced CTL activation has been achieved with 'anchor' residue modification of the melanoma antigen MART-1_{27-35} (AAGIGILTV) to LAGIGILTV [22]. Furthermore, mutations of the MART-1_{26-35} peptide epitope in the central position (positions 31-33) induced high affinity ligands to HLA-A2 and amplified the responses of a T cell MART-1_{26-35} specific clone [23]. The MUC1-8 peptide (SAPDTRPA) binds to murine MHC class I, H2-K^b with low affinity, of which the crystal structure is known [24]. Mutations to MUC1-8 peptide to result in MUC1-85F8L (SAPDFRPL) bound to H2-K^b with high affinity, induce high avidity CTL in C57BL/6 mice and overcame tolerance in MUC1 transgenic mice [25]. Recently, Ran1, tumor specific antigen CTL epitope was mutated to Ran1 1Y, where, position 1 was replaced with Y, elicited stronger CTL and bound to HLA-A2 with high affinity compared to wild type peptide [26]. Making anchor motif modifications to include high affinity anchors, does not necessarily indicate that the peptide will bind with high affinity to the MHC as one must consider proximal/distal amino acids [27]. Changes in the side chain of P8 of peptides TLTSCNTSL and TLTSCNTSV were shown to influence the orientation of Arg97 from the

HLA-A2 binding groove. Due to steric hindrance, Arg97 orientated towards the N-terminus of the peptide TLTSCNTSL, thus facilitating binding when a hydrogen bond donor/acceptor is placed at position P3. In contrast, Arg97 orientated towards the C-terminus when the small chain from valine was present at position P8, as for TLTSCNTSV. Anchor modifications may therefore be complicated in this manner and require rational alterations to peptide sequences, using molecular modeling techniques, in order to successfully achieve the desired effect. In an interesting study, the human Her-2/neu CTL peptide, [hHer-2/neu (435-443)] was injected into HDD transgenic mice [28]. The murine Her-2/neu (435-443) peptide [mHer-2/neu 9(435)] differs from the human peptide by one amino acid at position 4. The [hHer-2/neu (435-443)] peptide induced strong CTL against the murine peptide, and induced strong protective and therapeutic immunity against an adoptively transferred HLA-A2/Her-2/neu tumor [28]. Thus, enhanced immunity to the xenoantigen was induced compared to the self antigen in mice, and demonstrates that a one amino acid variation alters immune responses and overcomes tolerance in this mouse model. A number of T cell epitopes derived from infectious pathogens or pathogens which cause cancer are highly variable leading to peptides no longer capable of binding to MHC molecules or TCR thereby facilitating immune evasion. Naturally occurring peptide variants have been described in a number of diseases including viral diseases, malaria and cancer. One such example is of an EBV strain carrying an HLA A2-restricted epitope variant of LMP-1, which has been shown to be prevalent in nasopharyngeal carcinomas (NPCs). The variant has two natural mutations at anchor residues of an L to an F and an M to an I at positions 2 and 5 respectively[29]. The mutant peptide bound to HLA-A2 with lower affinity, CTL lysis was abrogated and IFN-gamma cytokine responses were not induced. These results show that EBV isolates from NPCs is dominated by an HLA A2-restricted natural variant of LMP-1, which would allow the virus to resist immune recognition and may in part contribute to the prevalence of NPC in these populations [29].

TCR contact Residue Modifications (Altered Peptide Ligands)

Peptides with amino acid substitutions which interact with the TCR are known as altered peptide ligands (APLs), Fig. (**1**). TAA are usually weakly immunogenic and their peptides bind to MHC class I with low affinity. APLs can be designed to increase their immunogenicity and to induce stronger T cell responses than the native peptide epitope, to act as a super-agonist. APLs may exhibit similar or optimal MHC anchor motifs and have equivalent MHC binding as the cognate peptide, however, APLs influence the type of potency of T cell responses by modulating intracellular signaling and phosphorylation patterns involved in T cell activation [30]. An example of APL is the HLA-A2 peptide (CAP1, YLSGANLNL) from carcinoembryonic antigen (CEA) overexpressed on colorectal, gastric and pancreatic cancers [31-32]. Substitution studies in the TCR contact residues, CAP1-6D (YLSGADLNL), significantly enhanced CAP1 specific CTL responses with unaltered MHC binding [33]. In initial clinical trials immunization with CAP1-6D, induced enhanced CTL responses in patients compared to CAP1 peptide [34]. However, in a recent clinical trial, CAP1-6D, generated low-affinity $CD8^+$ T cells which did not recognize CEA-expressing colorectal carcinoma cells, even though these T cells were reactive against the CAP1-6D peptide and to a lesser affinity to the CAP1 native peptide [35]. This study demonstrates, that further work is required to analyse tumor cross-recognition prior to any clinical usage of APL as anti-cancer vaccines. In addition, a single amino acid substitution of $MART1_{27-35}$ (AAGIGILTV) (substitution of Leu in position 1) enhances $MART1_{27-35}$ T cell activity, by inducing IFN-g and IL-2 *in vitro* and these T cells are insensitive to inhibitory effects of $MART1_{27-35}$ antagonist peptides [22]. Mutated $gp100_{209-217}$ peptides with preferred

HLA-A2 anchor residues bound with higher affinity to HLA-A2 as compared to the native gp100 peptide [36]. CTL lines generated from patients immunized with gp100 peptide showed enhanced IFN-gamma secretion in the presence of the mutated peptides[36]. CD4 T cells to lymphoblastic leukemia antigen TEL/AML proliferate specifically to TEL/AML as well as Th1 cytokine secretion are enhanced in the presence of APLs [37]. Thus, super-agonist APLs can be of use for anti-leukemic immunotherapy. Single mutations to a peptide are known to change the entire conformation of the peptide and thus, altering the conformation of peptide-MHC contact residues or the TCR contact residues. Modifications at anchor residues have been shown to dramatically influence the conformation of the MHC peptide-groove and have profound effects on TCR interactions [38]. It is important to be cautious in designing modifications to peptides for cancer immunotherapy to improve them of higher affinity, since this can effect T cell reactivity. To overcome these problems, solid-phase epitope recovery method has been used to determine reactive peptides with immunogenic properties of interest [39]. APLs have also been used (antagonists) for autoimmune diseases [40-46] and for infectious diseases [47]. More recently, molecular simulation was used to optimise the melanoma immunodominant epitope NY-ESO-1(157-165) by substituting TCR contact residues. A W to F substitution resulted in an enhanced ability to induce cross-reactive CTL responses with the wild type peptide and lysis of NY-ESO-1-expressing tumor cells [48]. Thus, APLs can be used in conjunction with molecular modeling/simulation and binding studies to enhance T cell responses.

MIMOTOPE VACCINE DESIGN

B Cell Mimotopes

Potential B cell epitopes may be identified using computer-based analysis of hydrophilicity, hydrophobicity, antigenic index, and surface probability based on the protein sequence [49-50]. Since there is only limited correlation between predicted and actual epitopes, epitope scanning methods such as PepScan with anti-sera need to be utilized [51-52]. Most vaccines are designed with the aim to generate cellular responses to destroy tumors. However, it is becoming increasingly evident that the presence of systemic antibodies that recognize tumor antigens such as MUC1 may offer a survival advantage in patients with cancer [53]. Passively administered naked mouse, human, chimeric or humanized antibodies that fix complement or have ADCC activity have shown some promise and the most impressive results have been generated with the antibodies, Trastuzumab (Herceptin®) and Rituximab (Rituxan®) [54-57]. An immunoconjugate of CD33 and calicheamycin has also given good responses in patients with acute myelogenic leukemia and is approved by the FDA[58].

As a means of generating antibodies to tumor associated proteins by active immunization, patients may be injected with tumor proteins with various adjuvants. However, in some cases responses are weak possibly due to tolerance to the protein [59]. An alternative approach that may generate greater antibody responses is to use antibody mimotopes. Mimotopes are peptides that bind to the paratope of antibodies. Mimotopes may be identified with the use of combinatorial synthetic peptide libraries or random peptide libraries displayed on bacteriophage [60-61]. In the latter approach peptide libraries are displayed fused to the bacteriophage coat proteins pIII or pVIII. Phage libraries with peptides of lengths 6-43 amino acids (aa) have been generated [62-63]. Using the process of successive panning on a particular target, specific bacteriophages that bind the target can be isolated. Since the phenotype of the phage is linked to the genotype, the specific peptide sequence of the specific phage can be identified by DNA sequencing. Several groups have identified mimotopes that

bind to the humanized anti-HER-2/neu antibody, Trastuzumab. The mimotope H98 (LLGPYELWELSH) was identified by panning a 12-mer peptide library with Trastuzumab as the target [64]. A GST-H98 fusion protein was able to block the binding of Trastuzumab to HER-2/neu and mice immunized with GST-H98 generated antibodies which bound to H98 as well as to HER-2/neu. In another study, 5 mimotopes of Trastuzumab were isolated from a constrained 10-mer library of which C-QMWAPQWGPD-C was the best candidate for immunogenicity studies [65]. Immunization of BALB/c mice with the constrained peptide resulted in antibody that bound to HER-2/neu expressing SK-BR-3 cells [65]. Three different anti-HER-2/neu antibodies (Ab2, Ab4 and Ab5) were used to pan against a 12-mer constrained (XCX_8CX) and a 15-mer linear random phage display library. Phage selected for binding to Ab2 bound Ab2 and not to Ab4 and Ab5. Some phage isolated from panning on Ab4 specifically reacted with Ab2 rather than Ab4 and some with Ab5 suggesting that the antibodies may have overlapping domains. All three peptides selected from the screening of the constrained library on Ab2 (VCQPWDHNSICN, SCQPWDAPARCE and HCLPRDRMGQCH) were capable of inhibiting the binding of Ab2 antibody to HER-2/neu expressing T47D cells [66]. Anti-CD20 antibody rituximab was panned with a phage library displaying 7-mer cyclic peptides and peptides with consensus sequence A(S)NPS was isolated [67]. The consensus sequence overlapped the CD20 ^{170}ANPS^{173} sequence and these peptides inhibited the binding of rituximab to CD20 +ve cells. Mice immunized with KLH-linked peptides generated sera that blocked rituximab binding to CD20 +ve cells. Interestingly, not all mimotopes were capable of generating specific antibodies and is influenced by the surrounding amino acids of the mimotope motif [68].

Mimotopes are extremely useful when tumor specific antibodies are available but the antigen is unknown or difficult to isolate and purify. An antibody against, MG7 antigen (Ag), a gastric cancer TAA, is used as a test for gastric cancer and to monitor treatment efficacy [69]. A linear 9-mer and cyclic 9-mer library were screened against the monoclonal antibody to MG7-Ag. Panning lead to groups of peptides sharing the consensus sequences $PLX_{0\text{-}2}S$, SAVR and XRMX. MHC class I and class II epitope prediction programs indicated that all the peptides could potentially bind several HLA molecules. A MG7-Ag mimotope (KPHVHTKGGGS) was incorporated with the universal T helper epitope, PADRE (AKFVAAWTLKAAZ) into a pcDNA3.1 plasmid for transfection into *Salmonella typhimurium* [70]. Oral administration of the *Salmonella* resulted in MG7-Ag specific antibody and partially protected mice from MG7-Ag expressing EAC tumor cells. Similar results were observed with an alternative oral vaccine utilizing a fusion gene of the mimotope to HbcAg gene [71]. Furthermore, a prime-boost vaccination strategy utilizing priming with the oral DNA vaccination followed by boosting with an adenovirus construct incorporating the MG7-Ag mimotope induced more efficient T cell responses and protection of mice from a tumor challenge [72]. A recent study identified peptide mimics of the melanoma cell-adhesion molecule (Mel-CAM) which when coupled to tetanus toxoid and injected into BALB/c mice induced antibodies which cross-reacted with Mel-CAM [73-74]. In addition, mimotopes of high-molecular weight melanoma-associated antigen (HMW-MAA) identified by screening a 9-mer phage library on anti-HMW-MAA antibody 225.28S share a consensus sequence which displays partial homology to the HMW-MAA. When the mimotope was fused to albumin binding protein and used to immunize mice, antibodies were generated that lyzed 518A2 melanoma cells in ADCC assays. Similar B-cell mimotopes have been identified for prostate specific membrane antigen, CAMPATH-1 (CD52), MUC1, CEA and Mgb1-Ag [75-77]. B cell mimotope identification is important for peptide based vaccines.

T Cell Mimotopes

Several strategies can be used to identify T cell epitopes from tumor associated antigens (TAA), (i) isolation of MHC molecules from tumor cells and eluted peptides fractionated by HPLC and identifying T cell stimulatory peptides by sequencing, (ii) Use of CTL clones and expression libraries from tumor cell cDNA and (iii) synthesizing overlapping peptides from known protein sequences and identifying peptides that stimulate antigen specific T cells. Similarly to the identification of B cell mimotopes using combinatorial libraries or phage display libraries, T cell mimotopes can be identified using antigen-specific T cell clones. To identify T cell stimulatory peptides using the combinatorial library approach, pools of 9 aa peptides are made that have each of the 9 positions randomized with the 19 aa (excluding Cys) and each position with a single aa and the other 8 positions randomized with the 19 aa to result in 171 pools. The target cells are pulsed with the pools and subjected to lysis by the CTL clone. Pools with the specific aa that cause lysis by the CTL defines that position with the particular aa. This method has been used to identify T cell mimotopes of the CD8+ T cell clone specific for cutaneous T cell lymphoma (CTCL) [78]. Two HLA-B8-restricted mimotopes PVKTKDIKL and PVKTKDIKL were identified and T cell lines were generated by stimulating peripheral blood mononuclear cells (PBMC) which were able to lyze autologous EBV pulsed cells as well as MyLa tumor cells. A mixture of these two mimotopes were used with tuberculin to immunize two HLA-B8 CTCL patients [79]. Mimotope specific T cell frequencies reached 0.21 and 0.52% in two patients and mimotope reactive T cells isolated from patients lyzed MyLa target cells and mimotope pulsed EBV-transformed B cells.

Peptide display libraries cannot be used to identify T cell mimotopes since T cells recognize peptides in complex with MHC class I. To enable this, single chain peptide MHC molecules have been expressed on phage *via* the pIII protein, on yeast and insect cells [80]. The most promising method is the baculovirus based system where a random peptide library of 9-10-peptides is linked to the N-terminus of β_2-microglobulin *via* a flexible linker expressed with membrane bound MHC class I H-$2D^d$ heavy chain [81]. The libraries can be screened with fluorescently labeled soluble $\alpha\beta$TCR using flow cytometry to identify T cell mimotopes. This method was used to identify a mimotope for a T cell, reactive to an unknown peptide in context with H-$2D^d$. The unknown peptide (AGATRWCRL) was identified from the homology of the mimotope (TGPTRWCRL) with a murine homolog of the *Drosophila* protein, spinster. A similar method has been used for baculovirus display of MHC class II I-A^b-peptide complex [82]. Interestingly the identified mimotope (FEAQRARRAARVD) for one of the TCRs had high homology to the peptide (FEAQKAKANKAV) used for immunization. T cell mimotope identification using libraries is a powerful method to use when designing peptide based vaccines.

Carbohydrate Mimotopes

Carbohydrate antigens as immunogens are not ideal being T cell independent antigens and induce IgM antibodies. Use of carrier proteins convert carbohydrate antigens to T cell dependent antigens [83-84]. The concept of using peptide mimotopes of carbohydrate is attractive because of the possibility of generating both cellular and humoral responses. Carbohydrates such as sialyl-Lewis X, Lewis X and Lewis Y are upregulated on a variety of cancers and vaccination against these with a carbohydrate mimotope is advantageous [85-86]. A peptide mimic (GGIYWRYDIYWRYDIYWRYD) of sialyl-Lewis X was identified and used as a Multiple Antigenic Peptide (MAP, see below, Fig. (**2**)) to immunize BALB/c mice [87-88]. A combination of IL-12 with MAP induced CTL in mice which protected

them against Lewis X expressing Meth A tumor cell challenge. In addition, the peptide DAHWESWL was identified as a mimic of the galα(1, 3)gal epitope by screening a peptide library on IB4 lectin [89-90]. This peptide and sugar was also mimicked by the MUC1 VNTR peptide APDTRPAPGS Fig. (**3**). Furthermore the CTL that recognized MUC1 also recognized the galα(1.3)gal mimic DAHWESWL, Fig. (**1**) [91-94]. Neuroblastoma and melanoma cells express the weakly immunogenic GD2 ganglioside that induces IgM antibody responses in patients. Currently, GD2 linked to KLH is used for active immunotherapy of melanoma [95]. Screening a 15-mer phage display library with the 14G2a anti-GD2 antibody resulted in several peptides of which peptide 47 (EDPSHSLGLDVALFM) had the greatest inhibition of 14G2a binding to $GD2^+$ neuroblastoma cells [96]. A more active analog 47-LDA (EDPSHSLGLDAALFM) was designed based on molecular modeling of peptide with 14G2a antibody. A DNAvaccine expressing the 47-LDA epitope inhibited the growth of GD2-expressing NSX neuroblastoma in mice [97].

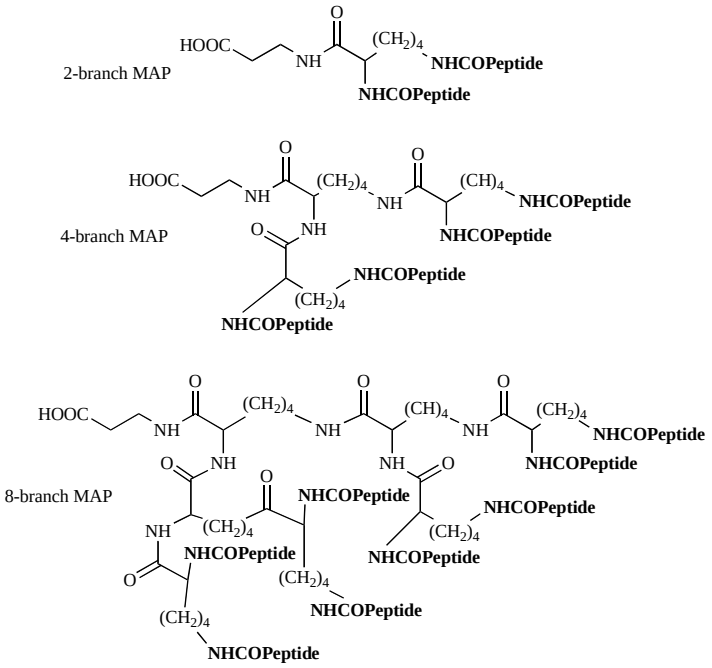

Fig. (2). Structure of 2-branch, 4-branch and 8-branch MAP for use in cancer vaccine studies.

Recently, it was demonstrated that the original 14G2a antibody crossreacted with the CD166 adhesion molecule and the CD8 T cells generated with the 47-LDA mimotope was recognizing a cross-reactive epitope within the CD166 molecule [98]. Carbohydrate, T cell and B cell mimotopes are great prerequisites in the design of peptide based vaccines for cancer immunotherapy.

MULTIEPITOPE VACCINES

An ideal vaccine for cancer encompass a means of overcoming tumor antigen escape variants, epitopes capable of priming MHC class I and class II responses and restricted to several HLA alleles. Use of several TAAs with a suitable adjuvant could satisfy this criteria.

However, development of such a vaccine may be hindered by the difficulty in being approved by regulatory agencies and possibly commercial licencing issues of antigens. In addition, it is likely that there will be a selective advantage for immunodominant epitopes to be presented in preference to the subdominant epitopes. The immunodominant epitopes would most likely be those of higher affinity and since tumor antigens are in most cases autoantigens and tolerance to self antigen may prevent the generation of strong immune responses. An elegant method to overcome these problems is to design a vaccine based on minimal CTL and T helper epitopes from one or more antigens. The so called 'polytope' vaccines can be made by genetic engineering methods or using chemical synthesis. In it's simplest form polytopes are made by synthesis of linear tandem peptides with or without spacer amino acids separating the minimal epitopes. The branched multiepitope peptide complexes have been most used in vaccines developed for infectious diseases but equally applicable to vaccines for cancer.

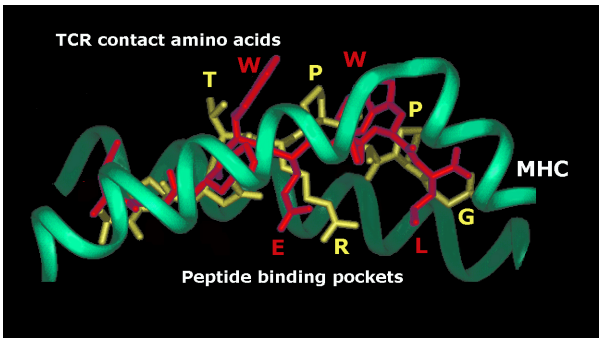

Fig. (3). Molecular model of MUC1 peptide (yellow; APDTRPAPG) and MUC1 mimic (red; DAWHESWL) in complex with MHC class I (green). In the model, a charged residue (R-P5 in the MUC1 peptide and E-P5 in the mimic peptide) point down into the groove of the MHC. All though, the amino acid sequence of the peptides (P4, P6/P7) vary at the TCR interface, the volume occupied by each of the amino acids is similar.

Linear multiepitopes linked in tandem

Within the sequence of human papillomavirus-16 antigen there is a continuous short peptide sequence that contains a CTL epitope (H-2D^b), T helper epitopes (I-A^b and I-E^b) and a pan specific B cell epitope ($E7_{44\text{-}62}$, QAEPDRAHYNIVTFCCKCD) [99]. Transcutaneous immunization of mice with the $E7_{44\text{-}62}$ peptide with CpG and cholera toxin protected mice from an E7 expressing tumor challenge [100]. Thymidylate synthetase (TS) is an intracellular enzyme overexpressed in cancer cells. Three HLA-A2 peptides were identified by MHC class I epitope prediction algorithms and binding to T2 cells. CTL lines were derived from HLA-A2 PBMC[101]. A 28-mer peptide was synthesized in tandem containing all three epitopes and mice were immunized which delayed the growth of TS transfected EL-4/HHD tumor [101]. In addition, the 20 aa tandem repeat sequence of MUC1 contains a B cell epitope (APDTR) and a number of CTL epitopes [102-104]. An oxidized mannan conjugate of a MUC1 GST fusion protein consisting of 5 tandem repeat sequences was highly immunogenic in mice and capable of eradicating MUC1 expressing tumors in C57BL/6, DBA/2 and BALB/c mice[105-144]. These conjugates [115-117]have been used in clinical trials by injection [118]or by *ex vivo* pulsed DC[119]. Amphipathic peptides such as fuseogenic peptides from HIV TAT protein, penetratin from *Drosophila Antennapedia* or cationic peptides

such as poly-arginine synthesized in tandem with CTL epitopes generate good cellular responses in mice[113, 120-123]. Lu and co-workers utilized synthetic peptides with the HIV-1 Tat-derived Trojan peptide in tandem with 3 different CTL epitopes to generate strong CTL responses to all three epitopes in mice when given with CpG [124]. Another amphipathic peptide pep-1 (KETWWETWWTEWSQPKKKRKV) non-covalently linked with peptides or proteins was used to facilitate transfer of a multi-epitope peptide consisting three HLA-A2 restricted epitopes Her-2/neu$_{369}$, Her-2/neu$_{435}$ and Her-2/neu$_{789}$ peptides separated by double arginine spacers across the cell membrane [125]. When administered in HHD HLA-A2 mice with a promiscuous T-helper epitope from tetanus toxoid and MDP primed CTL responses in mice 2-6 fold higher compared to the multiepitope peptide alone. The polycationic poly amino acid Poly-L-arginine simply mixed with TRP-2$_{181\text{-}188}$ H-2K^{b}-restricted tyrosinase-related protein 2 induced sustained T cell responses in mice [126]. Linear multiepitopes linked in tandem induce immune responses in mice and is a method being tested in human clinical trials.

Multiepitopes Linked in Branches *Via* a Poly-L-Lysine Core

Branched peptides known as Multiple Antigenic Peptides (MAP) was initially used for antibody production based on a lysine core capable of incorporating 4 or 8 B cell epitopes in one synthetic complex, Fig. (**2**). Since these are of high molecular weight they did not need any adjuvants for antibody production. The use of the MAP approach in cancer has not been as prevalent as it's use in vaccines for infectious diseases. The early MAPs had only identical peptides in either 4 or 8 branches [127]. However, using orthogonal protection schemes, MAPs with 2 or 3 different peptides can be incorporated into the complex [128]. Lipoproteins are components of bacterial cell walls and these have been shown to bind to TLR-2 [129]. The lipids are also involved in translocation of antigen into the cell. Lipid containing peptides can provide the necessary danger signals to prime effective immune responses. A lipid core peptide based on polylysine with 4 minimal CTL epitopes from either OVA$_{257\text{-}264}$ (SIINFEKL) or LCMV$_{33\text{-}41}$ and three C12 lipoamino acids activated DC (increased expression of CD86 and CD40) [130]. The lipid core peptide with SIINFEKL protected mice from an OVA positive tumor challenge but in combination with OVA to provide CD4 help.

A totally synthetic vaccine incorporating TLR-2 targeting lipid S-[2, 3-bis(palmitoyloxy) propyl]cysteine (Pam2Cys) with a T helper epitope, a CTL epitope and a B cell epitope [131], activated NF-kB-dependent gene activation *via* TLR-2 and matured DC. One particular construct incorporating the OVA CD8 epitope and a T helper epitope from the fusion protein of the morbillivirus canine distemper virus protected 50% of immunized mice from B16-OVA tumors [131]. Human malignant gliomas express a unique mutation in the gene that encodes the epidermal growth factor receptor. As a result of this mutation a unique epitope is expressed by gliomas and not on normal tissues which is a potential target for immunotherapy [132]. A MAP incorporating the unique epitope (LEEKKGNYVVTDHC) was used to immunize rats that generated a humoral response. Use of MAP with GM-CSF resulted in cellular responses (CTL, IFNγ) and reduced the growth of F98$_{EGFRvIII}$ tumors.

In the early studies MAPs were equipped with a B cell epitope because they were primarily designed for generating antibodies. However, for modern vaccines the ability to prepare MAPs with different multiple epitopes is desirable. By utilizing of orthogonally protected lysines, MAPs with B cell epitopes as well as a T helper epitopes have been synthesized [133]. Another approach to generate multiepitope constructs is by copolymerization of acryloyl peptides with acrylamide or another acryloyl aa [134-135]. This methodology enables the use of multiple acryloyl peptides to result in large molecular weight peptide poly-

mers. As an alternative to recombinant protein complex multiepitope proteins could be synthesized using orthogonal ligation strategies [136-138]. A multitude of chemical ligation procedures are available to selectively ligate prefunctionalized unprotected CTL, T helper or B cell epitopes, Fig. (**4A**). Several studies have also investigated the yield of ligation reaction, stability of the various linkages, the arrangement of epitopes and their immunogenicity [139]. In these studies epitopes ligated *via* disulphide linkages gave the lowest yields and were weakly immunogenic. Chemoselective ligation chemistry was used to design a branched oxime-linked peptide containing two copies of the MUC1 peptide and a universal T cell epitope, Fig. (**4A**) [140]. The 20 aa MUC1 sequence was incorporated in two orientations one with a free amino terminal and the other with a free carboxy terminal. A monomeric peptide and a linear dimeric peptide did not induce antibody responses but only the branched peptide with the oppositly oriented MUC1 peptide generated antibodies which reacted with the non-glycosylated MUC1.

In addition to peptides, carbohydrate antigens are also targets of immune responses. Tn, TF and sTn are carbohydrate antigens expressed on breast, prostate, lung and pancreatic cancers [141]. Vaccines that target these have been developed by conjugating the glycopeptides to carriers such as BSA, KLH or OSA. Novel scaffolds, regiospecifically addressable functionalized templates (RAFTs) can be used for design of these vaccines. RAFTs are composed of a backbone cyclized decapeptide with two proline-glycine β-turns and stabilizes the conformation in solution, Fig. (**4C**). A conjugate containing 4 Tn analogs and a CD4 T helper epitope from type I poliovirus (KLFAVWKITYKDT) was recognized by anti-Tn monoclonal antibodies indicating that the conjugate mimics the natural display of Tn antigens present on repeat S/T repeats of mucins [142]. Furthermore, the conjugates also stimulated poliovirus specific T cell hybridomas when presented by DC. By using suitable orthogonal protecting groups and/or chemoselective ligation procedures, the positions occupied by the B cell epitopes maybe replaced with other epitopes.

RECENTLY IDENTIFIED TAA CTL EPITOPES

Mesothelin is overexpressed in mesothelioma, pancreatic and ovarian cancers [143]. Two naturally expressed mesothelin specific HLA-A2 CTL have been demonstrated to lyze pancreatic and ovarian tumor cells *in vitro* [144]. In addition, agonist peptides of these HLA-A2 epitopes are able to bind with higher affinity to HLA-A2 molecules and the CTL lyze mesothelin expressing tumor cells more efficiently as well as induce increased numbers of specific CTLs *in vitro* from healthy individuals and cancer patients [144].

Adipophilin is a protein involved in lipid homeostasis of adipocytes and macrophages and is selectively overexpressed in some renal cell carcinoma (RCC) and more importantly is found at very low levels on normal tissues [145]. Only recently, CTLs were generated after pulsing with a previously identified HLA-A2+ restricted CTL epitope, SVASTITGV *in vitro* using DCs derived from HLA-A2+ healthy donors and T2 cells [146]which recognized endogenously expressed adipophilin protein in RCC, malignant melanoma, breast cancer, and multiple myeloma [147]. Moreover, CTL clones generated from chronic lymphocytic leukaemia (CLL) and plasma cell leukaemia patients with DCs pulsed with peptide SVASTITGV were able to lyze autologous leukaemia cells [147].

Papillomavirus binding factor (PBF) was recently identified using a cDNA expression cloning procedure as an osteosarcoma antigen recognized by autologous CTLs and a HLA-B55 restricted 12-mer peptide, CTACRWKKACQR [148]. Prevalence of this epitope is particularly low and much effort is required to identify HLA-A2 and HLA-24 restricted epitopes to be more clinically applicable.

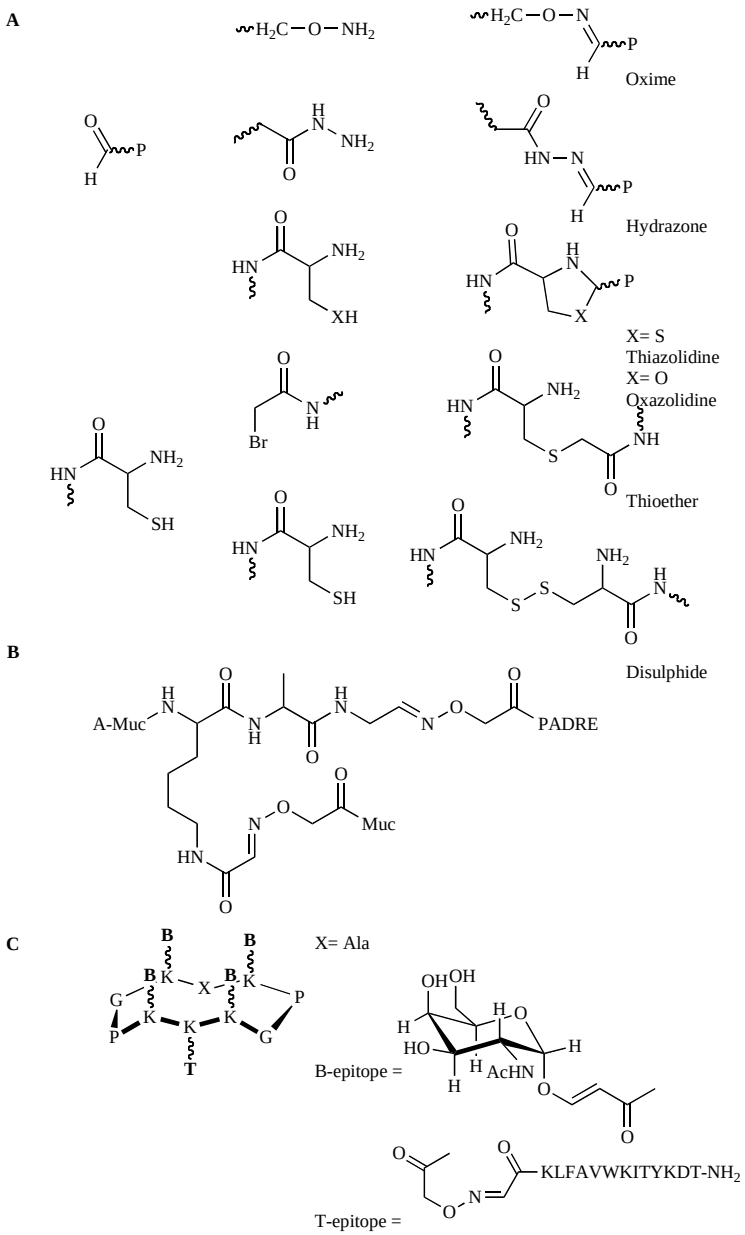

Fig. (4). A. Various chemoselective ligation strategies. **B**. Multiepitope peptide incorporating MUC1 and T helper epitopes. **C**. Example of a RAFT scaffold.

Ring finger protein 43 (RNF43) is expressed by colon cancer cells and HLA-A2 (ALWPWLLMA and ALWPWLLMAT) and HLA-A24 (NSQPVWLCL) restricted epitopes have been identified after generation of CTL clones [149]. These CTL clones were able to lyze targets pulsed with the specific peptides as well as tumor cells naturally expressing

RNF43 and it was clearly shown that the HLA-A24+ epitope induced enhanced tumor lysis compared to HLA-A2+ epitopes [149]. A more recent summary of recently identified T cell epitope peptides is shown in Table **1**.

Table 1. Summary of Newly Identified T Cell Epitopes

Antigen	Epitope	HLA Type	Refs
EGFR	853-861 ITDFGLAKL	HLA-A*201	[214]
HER-2/neu	828-836 QIAKGMSYL	HLA-A*201	[215]
Ribosomal protein L19	133-141 KNKRILMEH	HLA-A*31012	[216]
CEA	CEA.24, LLTFWNPPTTAKLTI CEA.488 RTTVKTITVSAELPK	HLA-DR	[217]
MUC4	P01204	HLA-A*0201	[218]
Papillomavirus binding factor	412-420 ALPSFQIPV	HLA-A*0201, HLA-A*0206	[219]
Cyclooxygenase-2	P479 479–487, ALYGDIDAV	HLA-A*0201 & HLA-A*03	[220]
CML66	70-78 WYQDSVYYI 76-84 YYIDTLGRI	HLA-A24 HLA-A*2402	[221-222]
MAGE-A4	284–293 YVKVLEHVVR; MAGE-A4 284–294 (YVKVLEHVVRV;	HLA-DPB1*0501 HLA-DRB1*1403-	[223]
NY-ESO-1	60-APRGPHGGAASG2	HLA-B7	[224]
HIF prolyl hydroxylase-3 (HIFPH3)	295-303 RYAMTVWYF	HLA-A24	[225]
PAX5	311-319 TLPGYPPHV	HLA-A2	[226]
Matrilysin (MMP-7)	96-107 SLFPNSPKWTSK	HLA-A3	[227]
Heparanase	525-533 (PAFSYSFFV, Hpa525), 277-285 (KMLKSFLKA, Hpa277), and 405-413 (WLSLLFKKL, Hpa405)	HLA-A2	[228]
Glypican-3	GPC3(144-152) FVGEFFTDV GPC3(298-306) EYILSLEEL	HLA-A*0201, HLA-A*2402	[229]
Cytochrome P450 1B1	CYP240 LVDVMPWLQY	HLA-A1, HLA-B35	[230]

CLINICAL TRIALS WITH PEPTIDE BASED VACCINES

Peptide vaccines incorporate defined tumor specific T cell epitopes. There are a number of newly defined CD8+ and CD4+ T cell epitopes derived from tumor antigens overexpressed on tumor cells being used for preclinical studies and are currently being used for the treatment of malignant melanoma as well as other cancers in Phase I, II and III clinical trials [150]. These vaccines attempt to induce activation and expansion of antigen-specific CD8+ and CD4+ T cells in the context of MHC class I and II molecules, respectively which can subsequently destroy tumor cells. Although peptide immunization often leads to the induction of strong T-cell responses, it has not been effective against established tumors in patients. A major challenge in developing peptide vaccines against cancer is breaking tolerance to tumor associated antigens which are self-proteins. In addition, sufficient CD4+ T helper responses are required for effective and lasting responses. To date most clinical trials have used MHC class I restricted peptides in combination with an adjuvant and no significant correlation between antigen specific CD8+ T cell expansion and the generation of protective immune responses has been shown (Table **2**-**4**).

Adjuvants

The identification of tumor antigens has spurred the development of more efficient adjuvants and novel delivery systems for cancer immunotherapy (summarized in Tables **2**-**4**). Immunologic adjuvants are used to generate effective tumor immunity by overcoming tolerance to tumor antigens which in most circumstances are self-antigens. GM-CSF acts as a growth factor to activate and stimulate DCs [151]. Intradermal immunization with GM-CSF results in activation and infiltration of DCs and enhances peptide-MHC complex formation [152]. GM-CSF has been shown in previous human clinical trials to enhance CD8+ T cell responses against tumor-specific epitopes such as tyrosinase and gp100 compared to incomplete Freund's adjuvant (IFA) [153, 154]. It has also been suggested that GM-CSF can enhance antigen processing and presentation of full length cancer proteins [155]. DC based immunization represents a promising approach for the immunotherapy of cancer. It was recently reported in a review by Banchereau and colleagues that after summarizing 6 melanoma based clinical trials using DCs loaded with melanoma antigen, resulted in total 9.5% tumor regression compared to 4.6% for other protocols including peptide vaccination, viral vectors and tumor cells [156]. However there are a number of considerations when using DCs as adjuvants in human clinical trials including the presence of diverse human DC subsets able to induce different immune responses *in vivo* [157-158], DC maturation [159], migration [160], antigen loading [160-162] and host related factors. The optimal conditions required to prepare DCs to induce the most effective and appropriate immune response remains to be defined. Bacterial DNA containing CpG motifs can trigger DCs [163]. CpG has also been shown to induce functional maturation of DCs loaded with tumor antigens and is effective in tumor vaccinations and has shown protection against lethal tumor challenges in mice [164]. Flt3 ligand (FL) is an important haemopoietic cytokine and injection of FL into humans has shown the most significant increases in DC precursors [165]. Numerous clinical trials have employed FL to increase DC numbers and some significant clinical responses have been observed [34]. There are currently many preclinical and clinical studies evaluating the potential of FL as an adjuvant for cancer immunotherapy. Interleukin 2 (IL-2) was first recognized as a promising immunotherapeutic agent after high dose administration of IL-2 in cancer patients resulted in tumor regression [166-168]. The action of IL-2 is different to previously discussed adjuvants as it does not have a direct effect on cancer cells but instead alters the host immune response and supports the effector functions induced by T cell responses [169]. Toll-like receptor (TLR) ligands have been shown to induce DC

maturation as well as enhance antigen presentation for activation of CTLs and natural killer (NK) cells. Numerous ligands for TLRs have been identified and are currently being used for clinical trials for melanoma. Imiquimod is a compound which binds to TLR-7 and TLR-8 and activates macrophages and DCs and stimulates secretions of pro-inflammatory cytokines [170]. A list of adjuvants and peptides used in human clinical trials in the last 2 years are summarized in (Table **1**, **2**).

Clinical Trials with Novel TAA Peptides

Numerous TAA have been identified and the immunogenicity of their MHC class I and class II epitopes identified and studied in preclinical and clinical trials. These have been extensively reviewed in the past literature [171]. In this section we will review preclinical and clinical studies of recently identified TAA (Table **2**-**4**).

NY-ESO-1

NY-ESO-1 is expressed by a broad range of human tumors including melanoma, breast, lung, prostate and bladder but not normal tissues except the testis and is considered the most immunogenic of the cancer testis antigens [172-173]. There has been several MHC class I but only a few class II restricted tumor epitopes identified in the NY-ESO-1 gene and immunization of these epitopes in humans can elicit antibody and CTL responses *in vivo* [174-178].

MHC class I and II restricted T cell epitopes have been identified by *in vitro* studies using overlapping long peptides spanning the NY-ESO-1 sequence. A number of new MHC class I restricted CTL epitopes have been identified recently and provide opportunities for new tumor associated antigens to be tested in preclinical and clinical studies. Two new epitopes (p92–104, LAMPFATPMEAEL) and (p94–102, MPFATPMEA) were identified after binding efficiently to HLA-B35 molecules and was presented and recognized by its corresponding polyclonal tumor infiltrating lymphocyte melanoma clone generated from melanoma invaded lymph nodes of stage III patients [179]. Peptide 92-104 was also presented by HLA-B51 whereas peptide 94-102 was presented by HLA-B51 as well as HLA-Cw3 [179].

Other MHC restricted CTL epitopes to NY-ESO-1 have been generated *in vitro*. A HLA-A24 restricted epitope, p158-166 was identified using a computer based epitope prediction program as well as using peptide binding assays with subsequent induction of specific CTLs from HLA-A24 healthy donors and the CTL *in vitro* killing of NY-ESO-1 expressing carcinomas. This finding was particulary important since HLA-A24 expressing individuals dominate in certain countries including Japan and provides additional tumor antigens to be used for peptide based vaccination [180]. NY-ESO-1 specific p158-166 was also shown to induce CTLs against colon carcinoma cell line WiDr, gastric carcinoma cell lines MKN7/28 and esophageal carcinoma cell line TE8 [180]. As well as identifying novel NY-ESO-1 specific tumor epitopes, immunodominant CTL epitopes are also been engineered to improve efficacy and reduce induction of heterogenous immune responses based on the x-ray crystallography structure of HLA-A2 complexed to NY-ESO p157-165. Two peptide analogues of NY-ES0 p157-165 were synthesized with the C-terminal cysteine residue was substituted to an alanine or a serine [181]. Substitution with alanine resulted in stability of the HLA-A2, NY-ESO p157-165 complex as well as maintaining specific CTL recognition from peptide vaccinated melanoma patients [181]. In neuroblastoma patients, NY-ESO-1-specific immune responses were observed for CD4+ and CD8+ T cells after *in vitro* stimulation with the HLA-A2 restricted peptide NY-ESO-1 p157-165 and were also able to recognize NY-ESO-1 expressing neuroblastoma cells [182].

Table 2. Summary of Immunological Adjuvants used in Peptide Based Clinical Trials from 2004-05

Adjuvants	Peptide Based Vaccine	Immunological Efficacy	Clinical Efficacy	Adverse Reactions	Refs
GM-CSF	HLA-A1, A2, A3 - restricted gp100 and tyrosinase peptides, tetanus helper peptide and montanide ISA-51	DTH, 67% antibody responses, 33% vaccine specific cell mediated responses	2 patients showed objective clinical responses 2 patients had stable disease	Vitiligo (15% patients)	[152]
	Pool of eight peptides derived from the complimentarity determining regions of human anti-p53 antibodies	All subjects had DTH to 2 or more peptides.Anti-peptide ab 4/6. No T cell response to p53.		No adverse reactions reported	[231]
DC	DCs pulsed with HLA-A2 or HLA-A2 restricted carcinoembryonic antigen peptides	70% CEA specific T cells	20% patients had stable disease for at least 12 weeks	No adverse reaction reported	[60]
	DCs were pulsed with CEA-derived, HLA-A24-restricted 9-mer peptide (CEA652)	Most patients showed DTH and positive *in vitro* CTL response to CEA652 peptide	Long term stable disease or marked decreases in the serum CEA level were observed in some patients	No adverse reactions reported	[232]
	DCs loaded with a cocktail of HLA-A2 restricted wild-type and modified p53 peptides	50% induced specific T cell responses against modified and unmodified p53 peptides	33% disease stabilization	No adverse reactions reported	[233]
	DCs loaded with HLA-A2 restricted hTERT 540 peptide (ILAKFLHWL) + KLH	57% showed hTERT specific CTL responses	Partial tumor regression in 1 patient	No adverse reactions reported	[234]
	DCs loaded with peptides from the melanoma antigens MAGE-3.A2, tyrosinase, gp100, and MART-1 + KLH	DTH	No clinical responses	No adverse reactions reported	[235]

Table 2 contd….

Adjuvants	Peptide Based Vaccine	Immunological Efficacy	Clinical Efficacy	Adverse Reactions	Refs
DC	DCs loaded with HLA-A1, A2, A3 restricted gp100 and tyrosinase peptides	11% and 13% peptide specific CTL in peripheral blood (PBL) and sentinel immunized node (SIN), respectively	1 patient showed objective clinical response	No adverse reaction reported	[152]
CpG 7909	CpG 7909 mixed with melanoma antigen A (Melan-A; identical to MART-1) analog peptide and IFA	100%patients exhibited rapid and strong antigen-specific T cell responses		No toxicity or adverse reactions reported	[236]
Flt3 ligand	Influenza (Flu), Melan-A (Mel), tyrosinase (Tyr), and NY-ESO-1 peptides + imiquimod+ Flt3 ligand	Increases in immature CD11c+ and CD123+ PMBC DCs	Pleiotropic clinical and hematological effects	Some patients developed clinically significant toxicities related to Flt3	[237]
	E75 HLA-A2 epitope from HER-2/neu + Flt3 ligand as a systemic vaccine adjuvant	Increased DC in peripheral blood DTH responses No significant peptide specific T-cell responses were detected by ELIspot		2 patients developed grade III skin reaction and grade II autoimmune hypothyroidism	[238]
IL-2	HLA-A1, A2, A3 restricted gp100 and tyrosinase peptides, tetanus helper peptide and IL-2 adminstered daily beginning day 7 (group1) or day 28(group2)	36% of peripheral blood and 38% of sentinenel immu-nized node in group 1, and in 53% of PBL and 83% of SINs in group 2.	Disease-free survival esti-mates at 2 years were 39% for group 1 and 50% for group 2.	No toxicity or adverse reactions reported	[239]
Interferon-alpha	Melan-A/MART-1:26-35(27L) and gp100:209-217 (210M)	5/7 enhancement of CD8+ anti-peptide responses. Increased frequency of effectors and effector memory cells.	3/7 stable disease.		[240]

Table 2 contd….

Adjuvants	Peptide Based Vaccine	Immunological Efficacy	Clinical Efficacy	Adverse Reactions	Refs
TLR ligands	Imiquimod, a Toll-like receptor-7 ligand with influenza (Flu), Melan-A (Mel), tyrosinase (Tyr), and NY-ESO-1 peptides + Flt3 ligand	Increased circulating peptide-specific CD8+ T-cells compared to Flt3 ligand		67% adverse reactions related to their cancer	[237]

Table 3. Summary of Peptide Based Vaccines used in Human Clinical Trials from 2004-05

Cancer Candidate	Type of Cancer	HLA Type	Adjuvant	Clinical Responses	Refs
Personalized peptide vaccine	Pancreatic cancer	HLA-A24 HLA-A2		7/11 DTH increased cellular and humoral responses	[241]
Melan A	Melanoma	8 HLA-A2	CpG 7909, incomplete Freund's adjuvant	Antigen specific CD8 T cells (>3%) Effector memory T cells – IFN gamma, granzyme B, perforin	[236]
Four gp100- and tyrosinase-derived peptides		HLA-A1 HLA-A2 HLA-A3	Tetanus helper peptide plus IL-2	IFNgamma responses in PBMC and sentinel lymph node (~40%) Disease free at 2 years ~39-50%	[239]
Individualized peptide vaccination	Prostate cancer	HLA-A24	Low dose estramustine	Decrease PSA DTH reaction at injection site	[242]
WT1 peptide-based immunotherapy	breast or lung cancer, myelodysplastic syndrome, or acute myeloid leukemia	HLA-A24	Montanide ISA51 adjuvant	18/26 received 3 or more vaccinations 12/20 showed reduction in tumor size, tumor markers, blast cells correlation between CTL and clinical responses	[243]
CTL precursor-oriented peptide vaccine	Advanced colorectal cancer	10 HLA-A24		5/10 patients showed increases antigen specific T cells 7/10 patients showed Aantipeptide IgG 3/10 DTH response 1/10 partial clinical response	[244]

Table 3. contd….

Cancer Candidate	Type of Cancer	HLA Type	Adjuvant	Clinical Responses	Refs
Pool of eight peptides derived from the complimentarity determining regions (CDRs) of human anti-p53 antibodies	Advanced malignancy	6/14 completed trial	GM-CSF	DTH Lots of antibodies and some cellular responses No T cell responses to p53	[231]
bcr-abl-derived fusion peptide vaccine	Chronic myelogenous leukaemia		Quillaja saponaria (QS-21)	14/14 DTH, CD4 proliferation 11/14 IFN gamma ELISPOT	[245]
ESO-1:165V (HLA-A2) NY-ESO-1:161-180 (HLA-DP4)	Metastatic melanoma	HLA-A2 HLA-DP4		Antigen specific T cells to ESO-1:165V more than NY-ESO-1:161-180	[189]
Influenza peptide NY-ESO-1 Melan A Tyrosinase	27 metastatic and high-risk melanoma		Flt3 ligand 8 patients had imiquimod (TLR7 ligand) applied topically	Increase CD11c+ and CD123+ Peptide specific CD8+ T cells in imiquimod vaccination patients	[237]
MUC1 peptide (100mer) (5xrepeat regions)	16 advanced pancreatic cancer		SB-AS2	Increase CD8+ T cells Increases total MUC1 antibody2 patients alive after 5 years	[246]
T-helper epitope derived from the melanoma differentiation antigen	Resected, high-risk metas-tatic melanoma	HLA-DR4		Th1 and Th2 T cell proliferation to epitope Secrete granzyme B, cytolysis	[247]
Carcinoembryonic antigen (CEA) peptides	10 late-stage colorectal carcinoma	HLA-A2 HLA-A24	Autologous DC + TNFalpha	Increase CEA specific T cells (70%) 2/10 stable disease for 12 weeks	[248]
CTL precursor-oriented peptide vaccine	10 advanced colorectal carcinoma	HLA-A24		5/10 patients showed increases antigen specific T cells 7/10 patients showed Aantipeptide IgG 3/10 DTH response 1/10 partial clinical response	[244]

Table 3. contd….

Cancer Candidate	Type of Cancer	HLA Type	Adjuvant	Clinical Responses	Refs
CEA652 9mer peptide	CEA-expressing metastatic gastrointestinal or lung adenocarcinomas	HLA-A24	Autologous DC (GM-CSF, IL-4)	DTH responses in most patients *In vitro* CTL response to CEA652 peptide after therapy	[232]
Cocktail of three wild-type and three modified p53 peptides	6 progressive advanced breast cancer	HLA-A2	Autologous DC	2/6 stable disease 3/6 specific T-cell responses against modified and unmodified p53 peptides	[233]
hTERT I540 peptide	Advanced breast and prostate carcinoma	HLA-A2	*Ex-vivo* DC and KLH	4/7 induced antigen specific responses 1/8 partial tumor regression	[234]
MAGE-3.A2, tyrosinase, gp100, and MART-1	14 AJCC stage IV melanoma		Non matured DC + KLH	5/14 stable disease 50% patients had responses to MART-1 peptide and a third to the other melanoma peptides	[235]

Table 4. Summary of Peptide Based Vaccines used in Human Clinical Trials

Cancer Candidate	Type of Cancer	HLA Type	Adjuvant	Clinical Responses	Refs
MAGE, MART-1/MelanA, gp100 and tyrosinase helper T-cell epitope pep-tides	Stage IIIB to IV melanoma	At least on HLA-DR1, -DR4, -DR11, -DR13 or –DR15	Montanide ISA-51	Proliferation responses to peptides in 81% patients.Objective clinical responses in 2/17 (1-3.9+ yrs), stable disease in 2/17 (1.8-4.6+ yrs).	[249]
CEA, HER2, p53, MAGE and PADRE (IDM-2101)	Non-small cell lung cancer	HLA-A2	Montanide ISA-51	One year survival was 60% and median survival time 17.3 months. 1 complete and 1 partial response. Survival longer in patients with an anti-peptide response	[250]

Table 4. contd….

Cancer Candidate	Type of Cancer	HLA Type	Adjuvant	Clinical Responses	Refs
Gp100, MART-1, tyrosinase, MAGE-3.A2, AMGE-A10 & NA17	Metastatic melanoma	HLA-A2	DC ± IL-2	Intranodal injection. 2/9 PR, 3 SD in groups receiving IL2 and 2 SD in groups witout IL-2. No difference in survival between groups that receive IL2 or not.	[251]
NY-ESO-1b	Ovarian cancer - after primary surgery and chemotherapy	HLA-A2	Montanide ISA51	3/4 NY-ESO-1+ve patients had T-cell immunity by tetramer & ELISpot, 6/9 media progression-free survival 13 months, 3/9 complete remission at 25, 38 and 52 months. T cell immunity in patients with or without NY-ESO-1 positive tumors.	[252]
WT1 peptide-based immunotherapy	Gynecological cancer	HLA-A24	Montanide ISA51 adjuvant	12 weekly injections 3/12 stable disease, 9/12 progressive disease	[253]
Her-2/neu	Metastatic breast cancer	Not restricted	Influenza virosomes	IM day 1, 28 & 56 8/10 increase peptide-specific antibody Increased IL-2, IFNgamma, TNFalpha	[254]
Her-2/neu (GP2)	Disease free, lymph node – ve breast cancer patients	HLA-A2	GM-CSF	Dose escalation. *In vivo* GP2-specific T cells increased from pre-vaccination: 0.8%-1.6%. Epitope spreading	[255]
HER2	Various metastatic tumors	B ell epitope and measles virus T cell epitope	Muramyl dipeptide/ SEPPIC ISA 720	Dose escalation.MTD 3 mg. 4 patients had stable disease, 2 partial responses & 11 progressive disease.	[256]
P53	Ovarian cancer	Not specified	Montanide ISA51	P53-specific T cells in all patients. T cells were $CD4^+$ and produced Th1 and Th2 cytokines.Stable disease evaluated by CA-125 and CT scans in 2/20.	[257]
Vascular endothelial growth factor 2	Metastatic pancreatic cancer	HLA-A*2402	Montanide ISA 51	Dose escalation. CTL in 11/18 patients. Median overall survival time = 8.7 months.	[258]

Table 4. contd….

Cancer Candidate	Type of Cancer	HLA Type	Adjuvant	Clinical Responses	Refs
HPV-16 E6/E7	Vulvar intraepithelial neoplasia	No restriction	Montanide ISA 51	At 12 months 15/19 had clinical responses with complete responses in 9/19.Strong CD4 and CD8 T cell responses in patients with complete response	[259]
Bcr-Abl fusion peptide	Chronic myeloid leukemia	No restriction	Montanide ISA 51	3/10 1-log reduction of Bcr-Able transcript levels	[260]
PSA, PSCA, PSMA, Survivin, Prostein, TRP, Flu matrix peptide	Prostate cancer-rising PSA but no detectable desease	HLA-A*0201	Montanide ISA51 + imiquimod, GM-CSF, mucin-1-mRNA/protamine, local hyperthermia or no adjuvant	Analysis based on PSA levels: 4/19 increased PSA, 2/11 stable PSA during vaccination, 3/19 decreased PSA (all received TLR-7 agonist), 11/19 increased PSA levels.	[261]
Mucin 1	Pancreatic & biliary cancer after resection of primary tumor.	No restriction	Dendritic cells	Transient increase of FoxP3+CD4+ cells after each immunization. 4/12 patients alive at 4-year follow up.	[262]
Survivin	Urothelial cancer	HLA-A24	Montanide ISA 51	Increased CTL precursor frequency in 5/9 patients. Small tumor reduction in 1 patient.	[263]
Survivin	Breast cancer	HLA-A*2402	± Montanide ISA 51	Increased peptide-specific CTL frequency in peptide + IFA groups. No clinical responses	[264]

CD4+ T cells play an important role in the induction and maintenance of adequate CD8+ T cell mediated anti-tumor responses. Therefore, identification of MHC class II restricted tumor antigenic epitopes is of major importance for the development of effective immunotherapies and has been subject to a great deal of study over the past few years. It was noted that polyclonal CD4+ T cell responses reacted to known and unknown NY-ESO-1 epitopes in 11 out the 13 melanoma patients tested while no healthy donors showed any responses [183]. The most immunogenic of the CD4+ epitopes tested was NY-ESO-1 peptide 80-109 containing restricted by HLA-DP4, HLA-DR, HLA-DR7 as well as a peptide showing diverse HLA class II binding [183-184]. Peptide 87–111 bound to HLA-DRB1 (DRB1-0101, DRB1-0401, DRB1-0701, DRB1-1101, DRB1-1501, DRB5-0101) [185]. In addition, p87–

111 and p87-101 was able to stimulate both HLA-DR and HLA-DP4 restricted CD4+ T cells from patient PBMCs. These patient specific NY-ESO-1 p87-111 and p87-101 CD4+ T cells were subsequently able to recognize autologous melanoma cell lines and autologous DCs loaded with NY-ESO-1 protein or transfected with NY-ESO-1 cDNA and stimulate both Th1 and Th2 CD4+ T cell responses [185]. Two novel CD4+ T cell epitopes were also identified after patients were immunized with monocyte derived dendritic cells pulsed with full-length NY-ESO-1 protein formulated with ISCOMATRIX [186]. HLA-DR2 restricted peptide (NY-ESO-1186–99) was dominant in one patient sample and suggested that the previously identified immunodominant HLA-DP4 restricted NY-ESO-1 p157-170 epitope was not the immunodominant epitope presented and others should be considered for inclusion in vaccine preparations [186]. Recently, CD4+ T cell clones were generated from a patient with NY-ESO-1 expressing synovial sarcoma by stimulation. These generated CD4+ clones were generated by peptide NY-ESO-1 p49-66 and p55-72 stimulation and was shown to be HLA-DQ B1 03011 restricted. Furthermore, the CD4+ clones stimulated with p49-66 showed cross-reactivity with p55-72 but not visa versa [187]. As well as reacting to the specific peptides, the NY-ESO-1 peptide reactive CD4+ T cell clones reacted against the naturally presented NY-ESO-1.

A clinical trial using peptides p157–167 (SLLMWITQCFL), p157–165 (SLLMWITQC), and p155–163 (QLSLLMWIT) in 33% DMSO and GM-CSF were injected in HLA-A2+ patients with progressing NY-ESO-1 expressing metastatic tumors of different types [175-177]. Both seronegative and seropositive patients induced antigen specific CD8+ IFN-γ T cell responses and developed DTH responses [175-177, 188]. There have been suggestions that synthetic peptide immunization derived from NY-ESO-1 may not be the most effective method of inducing anti-tumor responses in melanoma patients. Patients with metastatic melanoma injected with HLA-A2 restricted peptide, p165V, a HLA-DP4 restricted peptide p161-180 or both peptides given in parallel did not induce significant antigen specific CD8+ T cells with any of the vaccination regimes [189]. Subsequent clinical trials using NY-ESO-1 have used recombinant proteins and not peptides for melanoma patients. NY-ESO-1 protein formulated with the ISCOMATRIX adjuvant induced antibody responses, strong delayed-type hypersensitivity reactions and circulating NY-ESO-1 epitope specific CD8+ and CD4+ T cells [190]. A peptide based vaccine consisting of five defined melanoma associated peptides, including three overlapping NY-ESO-1 peptides, SLLMWITQCFL p157-167, SLLMWITQC p157-165, and QLSLLMWIT p155-163; as well as a tyrosinase internal peptide and Melan-A/MART-1 analogue peptide ELAGIGILTV in the presence of Flt3 ligand induced potent CD8+ and CD4+ T cells at the site of injection and the CD8+ T cells expanded and could also recognize natural tumor antigen, produce IFN-γ and kill in a cytotoxicity assay in patients with melanoma or resected stage II, III, or IV melanoma [191]. It is clear that NY-ESO-1 antigen is immunogenic in human clinical trials whether administered as protein or peptide.

Mammaglobin-A

Mammaglobin-A is a novel breast cancer associated antigen and is overexpressed in 80% of primary and metastatic breast tumors [192-193]. Levels of Mammaglobin-A are maintained among well, moderately and poorly differentiated breast tumors [194] and mammaglobin gene expression on leukapheresis products of high-risk breast cancer patients is an indicator of poor prognosis [195]. Only recently it was shown that mammaglobin-A can exist in two forms in breast tumor tissue and the high molecular weight form correlated negatively with tumor grade and proliferation rate [196]. Mammaglobin-A is largely restricted to mammary epithelium, thus, a clear understanding of T cell mediated immunity to

mammaglobin is important in designing specific peptide based vaccines against breast cancer [197]. Breast cancer patients have a significantly higher frequency of mammaglobin-A-reactive T cells as compared to normal individuals. Eight HLA-A3 restricted epitopes identified using MHC class I binding prediction program detected high (Mam-A3.3, Mam-A3.5, Mam-A3.6, Mam-A3.7) and low (Mam-A3.1, Mam-A3.2, Mam-A3.4, Mam-A3.8) affinity peptides [198]. CTL were observed against peptides low affinity peptides but not high affinity peptides. Mam-A3.1 was recognized as the immunodominant epitope [198]. In addition, HLA-A2 restricted epitopes have also been identified [199]. CTL recognized peptides Mam-A2.1, Mam-A2.2, Mam-A2.3, Mam-A2.4 and Mam-A2.7, however Mam-A2.2 was also recognized by T cells from healthy individuals [199]. When CTL lines were generated from HLA-A2 healthy individuals, they recognized MamA2.1, Mam-A2.2, Mam-A2.3 and Mam-A2.4 but not Mam-A2.7 and were able to kill UACC-812 breast cancer cell lines *in vitro*. DC transduced with a Tat-mammaglobin fusion protein stimulated antigen-specific CD4+ and CD8+ T cells in mice and further suggested the potential development of Mammaglobin-A as a vaccine candidate for breast cancer [200].

EphA2

EphA2 is a transmembrane receptor tyrosine kinase that is up-regulated on many aggressive tumor cells [201-202]. EphA2 is overexpressed and functionally altered on a number of cancers and promotes metastatic disease. In normal cells, EphA2 localizes to sites of cell-to-cell contact and functions as a negative regulator of cell growth [203]. EphA2 is an attractive candidiate for cancer immunotherapy as it is overexpressed on a number of tumors (breast, prostate, colon, lung) at high levels relative to surrounding epithelium and the highest levels are found on the most aggressive tumor cells [201, 202]. Interestingly, when EphA2 binds to its receptor, ephrinA1, it negatively regulates tumor cell growth and migration, however, EphA2 does not bind to its receptor on cancer cells and this does not affect its enzymatic activity [204]. Inhibitory antibodies have been designed to bind to EphA2 which results in its autophosphorylation and degradation and inhibits cancer cell growth [205].

In renal cell carcinoma (RCC) patients, CD4+ and CD8+ specific EphA2 T cell responses are detectable where the presence of CD8+ T cells is inversely correlated to the presence of disease in patients and increases after patients receive treatment [206]. CD4+ T cell responses have also been correlated with disease progression where the more advanced forms of RCC skew towards a Th2 response. Although the presence of CD8+ T cells in RCC was associated with disease-free stage, HLA-A2 restricted $EphA2_{883–891}$ specific CD8+ responses in RCC patients did not, however, it was noted that in 3 HLA-A2+ RCC patients with stage I disease, antigen-specific CD8+ T cells increased after curative surgery [206]. In addition, $EphA2_{663–677}$ specific Th1 specific CD4+ T cell responses were increased in Stage I RCC patients while Th2/Tr1 specific CD4+ T cell responses were consistently presence in Stage IV RCC patients [206]. Two HLA-A2-restricted epitopes ($EphA2_{58}$ and $EphA2_{550}$) have been described. These epitopes have a high affinity for HLA-A2, trigger CTL in HHD mice and *in vitro* in healthy humans and patients with prostate cancer [207]. Furthermore, in mouse models, immunization with H-$2K^b$-binding $mEphA2_{682–689}$, and I-A^b-binding $mEphA2_{30–44}$ loaded DC in C57BL/6 mice induced specific CTL lysis *in vitro*. Mice immunized with peptides, $EphA2_{671–679}$, $EphA2_{682–679}$, or $EphA2_{30–44}$ peptides showed protection against a EphA2 negative B16 tumor challenge and induced significant suppression of lung metastases after challenge with B16-BL6 tumor cells [208]. Mechanism of EphA2 protection was due to inhibition of vascular endothelial growth factor (VEGF) induced angiogenesis.

HCA587

The HCA587 gene belongs to MAGE-C subfamily with a large terminal exon encoding a protein of 373 aa and is highly expressed on human hepatocellular carcinoma (HCC) tissues [209]. HCA587 is not expressed on normal tissues except germ cells in testis and Purkinji cells in cerebellum. HCA587 protein is expressed on 37.1% from 71 HCC samples [210]. High levels of mRNA HCA587 have been detected in RCC patients [211]. It was also suggested that higher HCA587 protein levels correlated with poorly differentiated HCC tumors and HCA587 specific antibodies were only found in patients with poorly differentiated tumors [210]. Healthy individuals immunized with autologous DC loaded with HCA587 protein induce specific T cell responses [212]. HCA587-specific CD8+ and CD4+ T cell proliferation was detected as well as intracellular IFN-γ expression by PBMCs [212]. Six HCA587-derived peptides that bind to HLA-A2 have been described [213]. DC pulsed with high affinity binding HLA-A2 restricted HCA587 peptide p317-325, stimulated CD8+ T cells in the presence of IL-2 and IL-6 and generated HLA-A2 restricted CD8+ T cells against HCA587 p317-325 in healthy donors [213].

CONCLUSION

A number of tumor associated antigens have been characterized, with some of the more recent ones described here. It is clear that they all induce cellular and humoral responses in patients when delivered in an appropriate manner and are good targets for tumor immunotherapy studies. These antigens are either unique to a particular tumor type or are expressed at an elevated level on tumor cells compared to normal cells. There are also universal tumor antigens such as telomerase reverse transcriptase and survivin that are ideal targets particularly if combined with specific TAA. Many of these antigens or minimal epitopes together with a variety of adjuvants have been tested in preclinical studies and clinical studies. Nevertheless, the outcomes from the vaccine clinical trials is not impressive. Recently, much effort has been directed towards the identification of new TAA, using protein profiling, DNA arrays and prediction of epitopes *in silico*. Designing peptide based vaccines is a challenge, however, a number of delivery methods show promise in both preclinical and clinical settings. Synthetic chemical methods are available to design peptide vaccines incorporating various features to optimize immunogenicity such as minimal epitopes from multiple antigens (T helper, CTL and B cell epitopes, TLR activating compounds). Even the optimal vaccine may need to be administered with various cytokines that modulate T cell responses and overcome inhibitory signals in the microenvironment of tumors.

ABBREVIATIONS

aa	=	Amino acid
Ag	=	Antigen
APC	=	Antigen presenting cell
APL	=	Altered peptide ligand
CEA	=	Carcinoembrionic antigen
CTL	=	Cytotoxic T lymphocytes
DC	=	Dendritic cells
MAP	=	Multiple Antigenic Peptide
PBMC	=	Peripheral blood mononuclear cells

TAA = Tumor associated antigen

TLR = Toll-like receptor

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