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(57) Abrégé/Abstract:
The present invention provides HLA-DR (MHC class II) binding peptides derived from the ovarian/breast cancer associated antigens, Human Epidermal Growth Factor Receptor 2 (HER-2/neu), Carcinoembryonic Antigen (CEA), Insulin Growth Factor Binding Protein 2 (IGFBP-2), and Cyclin D1. The immunogenic peptides can be used in cancer vaccines.

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(54) Title: HLA-DR BINDING PEPTIDES AND THEIR USES

(57) Abstract: The present invention provides HLA-DR (MHC class II) binding peptides derived from the ovarian/breast cancer associated antigens, Human Epidermal Growth Factor Receptor 2 (HER-2/neu), Carcinoembryonic Antigen (CEA), Insulin Growth Factor Binding Protein 2 (IGFBP-2), and Cyclin D1. The immunogenic peptides can be used in cancer vaccines.

HLA-DR BINDING PEPTIDES AND THEIR USES

BACKGROUND OF THE INVENTION

Field of Invention

[0002] The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as cancers. In particular, it provides novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and induce an immune response.

[0003] MHC molecules are classified as either Class I or Class II molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, dendritic cells, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular immunogenic peptide that is displayed. Complexes between a particular disease-associated antigenic peptide and class II HLA molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of specific CTL and antibody immune responses.

[0004] A complex of an HLA molecule and a peptidic antigen acts as the ligand recognized by HLA-restricted T cells (Buus, S. *et al.*, *Cell* 47:1071, 1986; Babbitt, B. P. *et al.*, *Nature* 317:359, 1985; Townsend, A. and Bodmer, H., *Annu. Rev. Immunol.* 7:601, 1989; Germain, R. N., *Annu. Rev. Immunol.* 11:403, 1993).

[0005] Peptides of the present invention comprise epitopes that bind to HLA class II DR molecules. A greater degree of heterogeneity in both size and binding frame position of the motif, relative to the N- and C-termini of the peptide, exists for class II peptide ligands. This increased heterogeneity of HLA class II peptide ligands is due to the structure of the binding groove of the HLA class II molecule which, unlike its class I counterpart, is open at both ends. Crystallographic analysis of HLA class II

DRB*0101-peptide complexes showed that the major energy of binding is contributed by peptide residues complexed with complementary pockets on the DRB*0101 molecules. An important anchor residue engages the deepest hydrophobic pocket (*see, e.g.*, Madden, D.R. *Ann. Rev. Immunol.* 13:587, 1995) and is referred to as position 1 (P1). P1 may represent the N-terminal residue of a class II binding peptide epitope, but more typically is flanked towards the N-terminus by one or more residues. Other studies have also pointed to an important role for the peptide residue in the sixth position towards the C-terminus, relative to P1, for binding to various DR molecules.

[0006] In the past few years evidence has accumulated to demonstrate that a large fraction of HLA class I and class II molecules can be classified into a relatively few supertypes, each characterized by largely overlapping peptide binding repertoires, and consensus structures of the main peptide binding pockets. Thus, peptides of the present invention are identified by any one of several HLA-specific amino acid motifs, or if the presence of the motif corresponds to the ability to bind several allele-specific HLA molecules, a supermotif. The HLA molecules that bind to peptides that possess a particular amino acid supermotif are collectively referred to as an HLA "supertype."

[0007] Because human population groups, including racial and ethnic groups, have distinct patterns of distribution of HLA alleles it will be of value to identify motifs that describe peptides capable of binding more than one HLA allele, so as to achieve sufficient coverage of all population groups. The present invention addresses these and other needs.

[0008] T lymphocytes recognize an antigen in the form of a peptide fragment bound to the MHC class I or class II molecule rather than the intact foreign antigen itself. Antigens presented by MHC class II molecules are usually soluble antigens that enter the antigen presenting cell via phagocytosis, pinocytosis, or receptor-mediated endocytosis. Once in the cell, the antigen is partially degraded by acid-dependent proteases in endosomes. The resulting fragments or peptide associate with the MHC class II molecule after the release of the CLIP fragment to form a stable complex that is then transported to the surface for potential recognition by specific HTLs. *See* Blum, *et al.*, Crit. Rev. Immunol., 17: 411-17 (1997); Arndt, *et al.*, Immunol. Res., 16: 261-72 (1997).

[0009] Peptides that bind a particular MHC allele frequently will fit within a motif and have amino acid residues with particular biochemical properties at specific positions within the peptide. Such residues are usually dictated by the biochemical properties of the MHC allele. Peptide sequence motifs have been utilized to screen peptides capable of binding MHC molecules (Sette, *et al.*, *Proc. Natl. Acad. Sci. USA* 86:3296 (1989)), and it has previously been reported that class I binding motifs identified potential immunogenic peptides in animal models (De Bruijn, *et al.*, *Eur. J. Immunol.* 21: 2963-70 (1991); Pamer, *et al.*, *Nature* 353: 852-955 (1991)). Also, binding of a particular peptide to a MHC molecule has been correlated with immunogenicity of that peptide (Schaeffer, *et al.*, *Proc. Natl. Acad. Sci. USA* 86:4649 (1989)).

[0010] Accordingly, while some MHC binding peptides have been identified, there is a need in the art to identify novel MHC binding peptides from tumor associated antigens that can be utilized to generate an immune response in vaccines against these targets. Further, there is a need in the art to identify peptides capable of binding a wide array of different types of MHC molecules such they are immunogenic in a large fraction a human outbred population.

[0011] One of the most formidable obstacles to the development of broadly efficacious peptide-based immunotherapeutics has been the extreme polymorphism of HLA molecules. Effective coverage of a population without bias would thus be a task of considerable complexity if epitopes were used specific for HLA molecules corresponding to each individual allele because a huge number of them would have to be used in order to cover an ethnically diverse population. There exists, therefore, a need to develop peptide epitopes that are bound by multiple HLA antigen molecules at high affinity for use in epitope-based vaccines. The greater the number of HLA antigen molecules bound, the greater the breadth of population coverage by the vaccine. Analog peptides may be engineered based on the information disclosed herein and thereby used to achieve such an enhancement in breadth of population coverage.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIGURE 1 shows the identification of preexistent immunity to promiscuous HER-2/neu HLA-DR epitopes. Each panel shows a scatter gram of the mean

numbers of T cells specific for one of the identified HER-2/neu (see header of each panel) peptides in both healthy volunteer donors and patients. Each panel represents a unique peptide and each data point is derived from one individual. The grey box (i.e. cutoff) delineates the region that constitutes the mean and two standard deviations calculated from the normal healthy individuals. The percentages represent the fraction of patients that had mean T cell values above the cutoff. The peptides that are circled are those in which a higher fraction of the patients responded compared to the normal healthy controls. These peptides are considered to be the best vaccine candidates. However, it is clear that the rigidness of this approach could potentially result in many false negatives. For example, while p885 was shown to be recognized by 25% of patients, p886, a nearly identical peptide was found to be recognized by only 4%. However, if one looks at the graph for p886, there is a healthy individual that showed a robust response. The exclusion of that data point would have resulted in a patient response rate of 15%, which would have been consistent with the p885 response. Despite this, we identified 5 candidates to move forward. The fidelity of this approach is evident from a prior study which has already shown that a HER-2/neu peptide, p884-899, which encompasses the binding motif of p885, is an HLA-DR4 epitope.

[0013] FIGURE 2 shows the identification of preexistent immunity to promiscuous CEA HLA-DR epitopes. Figure 2 is identical to Figure 1 with the only exception that CEA is the antigen. Seven candidate peptides were identified.

[0014] FIGURE 3 shows the identification of preexistent immunity to promiscuous IGFBP2 HLA-DR epitopes. Figure 3 is identical to Figure 1 with the only exception that IGFBP2 is the antigen. Four candidate peptides were identified. Note that only 10 peptides were assessed as explained in the text above.

[0015] FIGURE 4 shows the identification of preexistent immunity to promiscuous IGFBP2 HLA-DR epitopes. Figure 4 is identical to Figure 1 with the only exception that Cyclin D1 is the antigen. Using the more liberal statistical method, 7 potential epitopes were identified.

[0016] FIGURE 5 shows the that HER-2/neu peptides, p59, p83, p88 and p885 are naturally processed and presented antigens. IFN-γ ELISpot analysis of short term T cell lines generated against HER-2/neu peptides, p53 (Panel A), p83 (Panel B), p88 (Panel C) and p885 (Panel D). The lines were tested for responses against respective

culture peptides, an irrelevant 15-mer peptide, a HER-2/neu protein fragment (amino acids 22-122, Panels A-C; amino acids 676-1255, Panel D), or an irrelevant similar weight protein, ovalbumin. Each shows the results from two lines established from two different breast or ovarian cancer patients who had a positive ELIspot response to the peptide. Each bar is the mean (s.e.m.) of three replicates.

[0017] FIGURE 6 shows that IGFBP2 peptides p17, p22, p249, and p293 are naturally processed peptides. Figure 6 is identical to Figure 5 except that the results were obtained using IGFBP-2 derived helper epitopes.

BRIEF SUMMARY OF THE INVENTION

[0018] The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as viral diseases and cancers. Thus, provided herein are novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and inducing or modulating an immune response. Some of the peptides disclosed are capable of binding human class II MHC (HLA) molecules, including HLA-DR and HLA-DQ alleles. Also provided are compositions that include immunogenic peptides having binding motifs specific for MHC molecules. The peptides and compositions disclosed can be utilized in methods for inducing an immune response, a helper T lymphocyte (HTL) response, or a cytotoxic T lymphocyte (CTL) response when administered to a system.

[0019] Epitopes on a number of immunogenic tumor associated antigens have been identified. The peptides are thus useful in pharmaceutical compositions for both in vivo and ex vivo therapeutic and diagnostic applications (*e.g.*, tetramer reagents; Beckman Coulter).

[0020] The peptides are also useful as epitope-based vaccines. The epitope-based vaccines preferably have enhanced, typically broadened, population coverage. The HLA-DR supermotif-bearing epitopes comprising the vaccine composition preferably bind to more than one HLA DR supertype molecule with a K_D of less than 1000nM or 500 nM, and stimulate a HTL response in patients bearing an HLA DR supertype allele to which the peptide binds.

[0021] Motif-bearing peptides may additionally be used as diagnostic, rather than immunogenic, reagents to evaluate an immune response. For example, an HLA-DR supermotif-bearing peptide epitope may be used prognostically to analyze an immune

response for the presence of specific HTL populations from patients who possess an HLA DR supertype allele bound by the peptide epitope.

[0022] The binding affinity of a peptide epitope in accordance with the invention for at least one HLA DR supertype molecule is preferably determined. A preferred peptide epitope has a binding affinity of less than 1000 nM, or more preferably less than 500 nM for the at least one HLA DR supertype molecule, and most preferably less than 50 nM.

[0023] Synthesis of a HLA DR supermotif-containing epitope may occur *in vitro* or *in vivo*. In a preferred embodiment, the peptide is encoded by a recombinant nucleic acid and expressed in a cell. The nucleic acid may encode one or more peptides, at least one of which is an epitope of the invention.

[0024] A peptide epitope of the invention, in the context of an HLA DR supertype molecule to which it binds, can be contacted, either *in vitro or in vivo*, with a cytotoxic T lymphocyte and thereby be used to elicit a T cell response in an HLA-diverse population.

[0025] An HTL epitope may be comprised by a single peptide. Further, the HTL epitope may be lipidated, preferably with palmitic acid, and may be linked by a spacer molecule to another HTL epitope or a CTL epitope. The epitope may be expressed by a nucleotide sequence; in a preferred embodiment the nucleotide sequence is comprised in an attenuated viral host.

[0026] As will be apparent from the discussion below, other embodiments of methods and compositions are also within the scope of the invention. Further, novel synthetic peptides produced by any of the methods described herein are also part of the invention.

[0027] The present invention provides peptides and nucleic acids encoding them for use in vaccines and therapeutics. The invention provides methods of inducing a helper T cell response against a preselected antigen in, a patient, the method comprising contacting a helper T cell with an immunogenic peptide of the invention. The peptides of the invention may be derived from a number of tumor associated antigens. The methods of the invention can be carried out in vitro or in vivo. In a preferred embodiment the peptides are contacted with the helper T cell by administering to the patient a nucleic acid molecule comprising a sequence encoding the immunogenic peptide.

[0028] The present invention is directed to methods of modulating the binding of peptide epitopes to HLA class II molecules. The invention includes a method of modifying binding of an original peptide epitope that bears a motif correlated with binding to an HLA molecule, said motif comprising at least one primary anchor position, said at least one primary anchor position having specified therefore primary anchor amino acid residues consisting essentially of two or more residues, said method comprising exchanging the primary anchor residue of the original peptide epitope for another primary anchor residue, with the proviso that the original primary anchor residue is not the same as the exchanged primary anchor residue. A preferred embodiment of the invention includes a method where the original primary anchor residue is a less preferred residue, and the exchanged residue is a more preferred residue.

[0029] One alternative embodiment of the invention includes a method of modifying binding of an original peptide epitope that bears a motif correlated with binding to an HLA molecule, said motif comprising at least one primary anchor position having specified therefore at least one primary anchor residue, and at least one secondary anchor position having specified therefore at least one secondary residue, said method comprising exchanging the secondary anchor residue of the original peptide epitope for another secondary anchor residue, with the proviso that the original secondary anchor residue is different than the exchanged amino acid residue. In some cases the original secondary residue is a deleterious residue and the exchanged residue is a residue other than a deleterious residue and/or the original secondary anchor residue is a less preferred residue and the exchanged residue is a more preferred residue.

[0030] As will be apparent from the discussion below, other methods and embodiments are also contemplated. Further, novel synthetic peptides produced by any of the methods described herein are also part of the invention.

[0030a] In accordance with an aspect of the present invention, there is provided a composition comprising the isolated HLA-DR binding peptide of SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109.

[0030b] In accordance with a further aspect of the present invention, there is provided the peptide of SEQ ID NO:145 in combination with one or more peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID

NO:109, for use as a diagnostic reagent to determine treatment, prognosis, or risk of cancer in a patient.

[0030c] In accordance with a further aspect of the present invention, there is provided a isolated polynucleotide that is a multi-epitope construct comprising between five and fifty-five nucleic acids each encoding an HTL epitope, wherein the HTL epitopes comprise SEQ ID NO:145 and one or more epitopes selected from the group consisting of SEQ ID NOS:86, 89, 90, and 109, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame.

[0030d] In accordance with a further aspect of the present invention, there is provided an isolated polynucleotide selected from the group consisting of:

(a) a multi-epitope construct comprising between five and fifty-five nucleic acids each encoding an HTL epitope, wherein the HTL epitope is any one of the epitopes listed in Table I and comprises SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame;

(b) a multi-epitope construct comprising between five and fifty-five nucleic acids each encoding an HTL epitope, wherein the HTL epitope is any one of the epitopes listed in Table II and comprises SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame;

(c) a multi-epitope construct comprising five and eight nucleic acids each encoding an HTL epitope, wherein the HTL epitope is any one of the epitopes listed in Table III and comprises SEQ ID NO:145 and SEQ ID NO:86, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame;

(d) the multi-epitope construct of (a) to (c), wherein one or more spacer nucleic acids are positioned between the HTL epitope nucleic acids of (a) or (b) or (c);

(e) the multi-epitope construct of (d), wherein said one or more spacer nucleic acids each encode 1 to 8 amino acids;

(f) the multi-epitope construct of (d), wherein one or more of said spacer nucleic acid encodes an amino acid sequence selected from the group consisting of: an amino acid sequence comprising or consisting of GPGPG (SEQ ID NO:193), an amino acid sequence comprising or consisting of PGPGP (SEQ ID NO:194), an amino acid sequence comprising or

consisting of (GP)n, an amino acid sequence comprising or consisting of (PG)n, an amino acid sequence comprising or consisting of (GP)nG, and an amino acid sequence comprising or consisting of (PG)nP, where n is an integer between zero and eleven;

(g) the multi-epitope construct of any one of (a) to (f), further comprising one or more MHC Class II targeting nucleic acids;

(h) the multi-epitope construct of (g), wherein said one or more targeting nucleic acids encode one or more targeting sequences selected from the group consisting of : an Ig kappa signal sequence, a tissue plasminogen activator signal sequence, an insulin signal sequence, an endoplasmic reticulum signal sequence, a LAMP-1 lysosomal targeting sequence, a LAMP-2 lysosomal targeting sequence, an HLA-DM lysosomal targeting sequence, an HLA-DM-association sequence of HLA-DO, an Ig-a cytoplasmic domain, an Ii protein, an influenza matrix protein, an HCV antigen, and a yeast Ty protein;

(i) the multi-epitope construct of any one of (a) to (h), wherein the construct is optimized for HTL epitope processing;

(j) the multi-epitope construct of any one of (a) to (i), wherein said HTL nucleic acids are sorted to minimize the number of HTL junctional epitopes encoded therein;

(k) the multi-epitope construct of any one of (a) to (j), wherein said multi-epitope construct comprises at least two Carcinoembryonic Antigen (CEA) HTL epitopes, at least two Cyclin D1 HTL epitopes, at least two Human Epidermal Growth Factor Receptor 2 (HER-2/neu) HTL epitopes, and at least two Insulin Growth Factor Binding Protein 2 (IGFBP-2) HTL epitopes;

(l) the multi-epitope construct of (k), wherein at least two Carcinoembryonic Antigen (CEA) HTL epitopes, at least two Cyclin D1 HTL epitopes, at least two Human Epidermal Growth Factor Receptor 2 (HER-2/neu) HTL epitopes, and at least two Insulin Growth Factor Binding Protein 2 (IGFBP-2) HTL epitopes are selected from the epitopes listed in Table II; and

(m) the multi-epitope construct of any one of (a) to (l), wherein said multi-epitope construct consists of the epitopes listed in Table III.

[0030e] In accordance with a further aspect of the present invention, there is provided an isolated nucleic acid encoding the isolated HLA-DR binding peptide of SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ

ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, and at least one protein or polypeptide included to enhance or decrease immunogenicity, wherein the at least one protein or polypeptide is a cytokine, a cytokine-inducing molecule, or a costimulatory molecule.

Definitions

[0031] The following definitions are provided to enable one of ordinary skill in the art to understand some of the preferred embodiments of invention disclosed herein. It is understood, however, that these definitions are exemplary only and should not be used to limit the scope of the invention as set forth in the claims. Those of ordinary skill in the art will be able to construct slight modifications to the definitions below and utilize such modified definitions to understand and practice the invention disclosed

herein. Such modifications, which would be obvious to one of ordinary skill in the art, as they may be applicable to the claims set forth below, are considered to be within the scope of the present invention. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in patents, published patent applications and other publications and sequences from GenBank and other databases.

[0032] An "HLA supertype or family", as used herein, describes sets of HLA molecules grouped on the basis of shared peptide-binding specificities, rather than serologic supertypes based on shared antigenic determinants. HLA class II molecules that share somewhat similar binding affinity for peptides bearing certain amino acid motifs are grouped into HLA supertypes. The terms "HLA superfamily," "HLA supertype family," "HLA family," and "HLA xx-like molecules" (where xx denotes a particular HLA type), are synonyms.

[0033] As used herein, the term "IC_{50}" refers to the concentration of peptide in a binding assay at which 50% inhibition of binding of a reference peptide is observed. Depending on the conditions in which the assays are run (*i.e.*, limiting MHC proteins and labeled peptide concentrations), these values may approximate K_D values. It should be noted that IC_{50} values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (e.g., HLA preparation, etc.). For example, excessive concentrations of HLA molecules will increase the apparent measured IC_{50} of a given ligand.

[0034] Alternatively, binding is expressed relative to a reference peptide. As a particular assay becomes more, or less, sensitive, the IC_{50}'s of the peptides tested may change somewhat. However, the binding relative to the reference peptide will not change. For example, in an assay run under conditions such that the IC_{50} of the reference peptide increases 10-fold, the IC_{50} values of the test peptides will also shift approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder is generally based on its IC_{50}, relative to the IC_{50} of a standard peptide.

[0035] As used herein, "high affinity" with respect to peptide binding to HLA class II molecules is defined as binding with an K_D (or IC_{50}) of less than 50nM. "Intermediate affinity" is binding with a K_D (or IC_{50}) of between about 50 and about 500nM. As

used herein, "high affinity" with respect to binding to HLA class II molecules is defined as binding with an K_D (or IC_{50}) of less than 100 nM. "Intermediate affinity" is binding with a K_D (or IC_{50}) of between about 100 and about 1000 nM. Assays for determining binding are described in detail, *e.g.*, in PCT publications WO 94/20127 and WO 94/03205.

[0036] Binding may also be determined using other assay systems including those using: live cells *(e.g.,* Ceppellini *et al., Nature* 339:392 (1989); Christnick et *al., Nature* 352:67 (1991); Busch *et al., Int. Immunol.* 2:443 (1990); Hill *et al..,* J *Immunol.* 147:189 (1991); del Guercio *et al.,* J *Immunol.* 154:685 (1995)), cell free systems using detergent lysates *(e.g.,* Cerundolo *et al.,* J *Immunol.* 21 :2069 (1991)), immobilized purified MHC *(e.g.,* Hill *et al.,* J *Immunol.* 152,2890 (1994); Marshall *et al.,* J *Immunol.* 152:4946 (1994)), ELISA systems *(e.g.,* Reay *et* al., *EMBO* J 11 :2829 (1992)), surface plasmon resonance *(e.g.,* Khilko *et al.,* J *Biol. Chem.* 268:15425 (1993)); high flux soluble phase assays (Hammer *et al., J. Exp. Med.* 180:2353 (1994)).

[0037] The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. In certain embodiments, the oligopeptides of the invention are less than about 50 residues in length and usually consist of between about 6 and about 25 residues, preferably 14 or 15 residues. Further, an oligopeptide of the invention can be such that it does not comprise more than 50 contiguous amino acids of a native antigen. The preferred HTL-inducing peptides of the invention are 30 residues or less in length, sometimes 20 residues or less and usually consist of between about 6 and about 25 residues, preferably 14 or 15 residues.

[0038] "Synthetic peptide" refers to a peptide that is not naturally occurring, but is man-made using such methods as chemical synthesis or recombinant DNA technology.

[0039] The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown,

are in the form they would assume at physiologic pH values, unless otherwise specified. In the amino acid structure formula, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids having D-forms is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G. Symbols for each amino acids are shown below:

Table 1

Amino acids with their abbreviations

Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[0040] With regard to a particular amino acid sequence, an "epitope" is a set of amino acid residues which is involved in recognition by a particular immunoglobulin, or in the context of T cells, those residues necessary for recognition by T cell receptor proteins and/or Major Histocompatibility Complex (MHC) receptors. In an immune system setting, *in vivo* or *in vitro,* an epitope is the collective features of a molecule, such as primary, secondary and tertiary peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor or HLA molecule. Throughout this disclosure epitope and peptide are often used interchangeably.

[0041] It is to be appreciated that protein or peptide molecules that comprise an epitope of the invention as well as additional amino acid(s) are still within the bounds of the invention. In certain embodiments, there is a limitation on the length of a peptide of the invention. The embodiment that is length-limited occurs when the protein/peptide comprising an epitope of the invention comprises a region (i.e., a contiguous series of amino acids) having 100% identity with a native sequence. In order to avoid the definition of epitope from reading, *e.g.,* on whole natural molecules, there is a limitation on the length of any region that has 100% identity with a native peptide sequence. Thus, for a peptide comprising an epitope of the invention and a region with 100% identity with a native peptide sequence, the region with 100% identity to a native sequence generally has a length of: less than or equal to 600 amino acids, often less than or equal to 500 amino acids, often less than or equal to 400 amino acids, often less than or equal to 250 amino acids, often less than or equal to 100 amino acids; often less than or equal to 85 amino acids, often less than or equal to 75 amino acids, often less than or equal to 65 amino acids, and often less than or equal to 50 amino acids. In certain embodiments, an "epitope" of the invention is comprised by a peptide having a region with less than 51 amino acids that has 100% identity to a native peptide sequence, in any increment down to 5 amino acids.

[0042] Accordingly, peptide or protein sequences longer than 600 amino acids are within the scope of the invention, so long as they do not comprise any contiguous sequence of more than 600 amino acids that have 100% identity with a native peptide sequence. For any peptide that has five contiguous residues or less that correspond to a native sequence, there is no limitation on the maximal length of that peptide in order to fall within the scope of the invention. It is presently preferred that a CTL epitope be less than 600 residues long in any increment down to eight amino acid residues.

[0043] A "dominant epitope" induces an immune response upon immunization with whole native antigens which comprise the epitope. (See, *e.g.*, Sercarz, *et al.*, *Annu. Rev. Immunol.* **11**:729-766 (1993)). Such a response is cross-reactive *in vitro* with an isolated peptide epitope.

[0044] A "cryptic epitope" elicits a response by immunization with isolated peptide, but the response is not cross-reactive *in vitro* when intact whole protein which comprises the epitope is used as an antigen.

[0045] A "subdominant epitope" is an epitope which evokes little or no response upon immunization with whole antigens which comprise the epitope, but for which a response can be obtained by immunization *in vivo* or *in vitro* with an isolated epitope, and this response (unlike the case of cryptic epitopes) is detected when whole protein is used to recall the response *in vitro*.

[0046] A "pharmaceutical excipient" comprises a material such as an adjuvant, a carrier, pH-adjusting and buffering agents, tonicity adjusting agents, wetting agents, preservatives, and the like.

[0047] As used herein, the term "pharmaceutically acceptable" refers to a generally non-toxic, inert, and/or physiologically compatible composition.

[0048] As used herein, the term "protective immune response" or "therapeutic immune response" refers to a HTL and/or a CTL response to a tumor associated antigen, which in some way prevents or at least partially arrests disease symptoms, side effects or progression. The immune response may include an antibody response that has been facilitated by the stimulation of helper T cells.

[0049] In certain embodiments, an "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will bind an MHC (HLA) molecule and induce a HTL response. Immunogenic peptides of the invention are capable of binding to an appropriate class II MHC molecule (*e.g.*, HLA-DR) and inducing a helper T cell response against the antigen from which the immunogenic peptide is derived.

[0050] An "immunogenic response" includes one that stimulates a HTL and/or CTL response *in vitro* and/or *in vivo* as well as modulates an ongoing immune response through directed induction of cell death (or apoptosis) in specific T cell populations.

[0051] Immunogenic peptides of the invention are capable of binding to an appropriate HLA-DR molecule and inducing a helper T-cell response against the

antigen from which the immunogenic peptide is derived. The immunogenic peptides of the invention are less than about 50 residues in length, often 30 residues or less in length, or 20 residues or less in length and usually consist of between about 6 and about 25 residues, preferably 14 or 15 residues.

[0052] The term "derived" when used to discuss an epitope is a synonym for "prepared." A derived epitope can be isolated from a natural source, or it can be synthesized in accordance with standard protocols in the art. Synthetic epitopes can comprise artificial amino acids "amino acid mimetics," such as D isomers of natural occurring L amino acids or non-natural amino acids such as cyclohexylalanine. A derived/prepared epitope can be an analog of a native epitope.

[0053] Immunogenic peptides are conveniently identified using the binding motif algorithms described for the specific HLA subtype (*e.g.*, HLA-DR). The algorithms are mathematical procedures that produce a score which enables the selection of immunogenic peptides. Typically one uses the algorithmic score with a "binding threshold" to enable selection of peptides that have a high probability of binding at a certain affinity and will in turn be immunogenic. The algorithm is based upon either the effects on MHC binding of a particular amino acid at a particular position of a peptide or the effects on binding of a particular substitution in a motif containing peptide.

[0054] The term "residue" refers to an amino acid or amino acid mimetic incorporated into an oligopeptide by an amide bond or amide bond mimetic.

[0055] A "conserved residue" is an amino acid which occurs in a significantly higher frequency than would be expected by random distribution at a particular position in a peptide. Typically a conserved residue is one where the MHC structure may provide a contact point with the immunogenic peptide. At least one to three or more, preferably two, conserved residues within a peptide of defined length defines a motif for an immunogenic peptide. These residues are typically in close contact with the peptide binding groove, with their side chains buried in specific pockets of the groove itself. Typically, an immunogenic peptide will comprise up to three conserved residues, more usually two conserved residues.

[0056] The term "motif" refers to the pattern of residues in a peptide of defined length, usually about 6 to about 25 amino acids, which is recognized by a particular MHC allele (one or more HLA molecules). The peptide motifs are typically different

for each human MHC allele and differ in the pattern of the highly conserved residues and negative residues. Peptide motifs are often unique for the protein encoded by each human HLA allele, differing in their pattern of the primary and secondary anchor residues. Typically as used herein, a "motif" refers to that pattern of residues which is recognized by an HLA molecule encoded by a particular allele. The binding motif for an allele can be defined with increasing degrees of precision.

[0057] The designation of a residue position in an epitope as the "carboxyl terminus" or the "carboxyl terminal position" refers to the residue position at the end of the epitope which is nearest to the carboxyl terminus of a peptide, which is designated using conventional nomenclature as defined below. The "carboxyl terminal position" of the epitope may or may not actually correspond to the end of the peptide or polypeptide.

[0058] The designation of a residue position in an epitope as "amino terminus" or "amino-terminal position" refers to the residue position at the end of the epitope which is nearest to the amino terminus of a peptide, which is designated using conventional nomenclature as defined below. The "amino terminal position" of the epitope may or may not actually correspond to the end of the peptide or polypeptide.

[0059] A "motif bearing peptide" or "peptide which comprises a motif" refers to a peptide that comprises primary anchors specified for a given motif or supermotif.

[0060] In certain embodiments, a "supermotif" is a peptide binding specificity shared by HLA molecules encoded by two or more HLA alleles. Preferably, a supermotif-bearing peptide is recognized with high or intermediate affinity (as defined herein) by two or more HLA molecules or antigens.

[0061] Alternatively, the term "supermotif" refers to motifs that, when present in an immunogenic peptide, allow the peptide to bind more than one HLA antigen. The supermotif preferably is recognized with high or intermediate affinity (as defined herein) by at least one HLA allele having a wide distribution in the human population, preferably recognized by at least two alleles, more preferably recognized by at least three alleles, and most preferably recognized by more than three alleles.

[0062] "Human Leukocyte Antigen" or "HLA" is a human class I or class II Major Histocompatibility Complex (MHC) protein (*see*, Stites, *et al.,* IMMUNOLOGY, 8TH ED., Lange Publishing, Los Altos, CA (1994).

[0063] "Major Histocompatibility Complex" or "MHC" is a cluster of genes which plays a role in control of the cellular interactions responsible for physiologic immune responses. In humans, the MHC complex is also known as the HLA complex. For a detailed description of the MHC and HLA complexes, see, Paul, FUNDAMENTAL IMMUNOLOGY, 3RD ED., Raven Press, New York, 1993.

[0064] The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of this invention do not contain materials normally associated with their in situ environment, e.g., MHC class II molecules on antigen presenting cells. Even where a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

[0065] "Peripheral blood mononuclear cells" (PBMCs) are cells found in from the peripheral blood of a patient. PBMCs comprise, e.g., CTLs and HTLs and antigen presenting cells. These cells can contact an antigen *in vivo*, or be obtained from a mammalian source and contacted with an antigen *in vitro*.

[0066] "Cross-reactive binding" indicates that a peptide is bound by more than one HLA molecule; a synonym is degenerate binding.

[0067] "Promiscuous recognition" is where the same peptide bound by different HLA molecules is recognized by the same T cell clone. It may also refer to the ability of a peptide to be recognized by a single T cell receptor in the context of multiple HLA alleles.

[0068] "Link" or "join" refers to any method known in the art for functionally connecting peptides, including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, and electrostatic bonding.

[0069] A "non-native" sequence or "construct" refers to a sequence that is not found in nature, *i.e.*, is "non-naturally occurring". Such sequences include, *e.g.*, peptides that are lipidated or otherwise modified, and polyepitopic compositions that contain epitopes that are not contiguous in a native protein sequence.

[0070] As used herein, a "vaccine" is a composition that contains one or more peptides of the invention, see, *e.g.*, TABLE I. There are numerous embodiments of

vaccines in accordance with the invention, such as by a cocktail of one or more peptides; one or more peptides of the invention comprised by a polyepitopic peptide; or nucleic acids that encode such peptides or polypeptides, *e.g.,* a minigene that encodes a polyepitopic peptide. The "one or more peptides" can include any whole unit integer from 1-150, *e.g.*, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 , 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 or more peptides of the invention. The peptides or polypeptides can optionally be modified, such as by lipidation, addition of targeting or other sequences. HLA class II-binding peptides of the invention can be linked to HLA class I-binding peptides, to facilitate activation of both cytotoxic T lymphocytes and helper T lymphocytes. Vaccines can comprise peptide pulsed antigen presenting cells, *e.g.,* dendritic cells.

DETAILED DESCRIPTION OF THE INVENTION

[0071] Certain embodiments of the present invention relate in part to an epitope-based approach for vaccine design. Such an approach is based on the well-established finding that the mechanism for inducing HTL immune response comprises the step of presenting a HTL epitope as a peptide of about 6-25 amino acids bound to an HLA molecule displayed on an antigen-presenting cell.

[0072] Certain embodiments of the present invention relate to peptides comprising allele-specific peptide motifs and supermotifs which bind to HLA class II molecules.

[0073] As noted above, high HLA binding affinity is correlated with higher immunogenicity. Higher immunogenicity can be manifested in several different ways. For instance, a higher binding peptide will be immunogenic more often. Close to 90% of high binding peptides are immunogenic, as contrasted with about 50% of the peptides which bind with intermediate affinity. A higher binding peptide will also lead to a more vigorous response. As a result, less peptide is required to elicit a similar biological effect. Thus, in some embodiments of the invention high binding epitopes are particularly desired.

[0074] It has been noted that a significant number of epitopes derived from known non-viral tumor associated antigens (TAA) bind HLA Class II with intermediate affinity (IC_{50} in the 50-500 mM range). It has been found that 8 of 15 known TAA

peptides recognized by tumor infiltrating lymphocytes (TIL) or CTL bound in the 50-500 mM range. These data are in contrast with estimates that 90% of known viral antigens that were recognized as peptides bound HLA with IC_{50} of 50 mM or less while only approximately 10% bound in the 50-500 mM range (Sette, et al., J. Immunol., 153:5586-5592 (1994)). This phenomenon is probably due in the cancer setting to elimination, or functional inhibition of the CTL recognizing several of the highest binding peptides, presumably because of T cell tolerization events.

[0075] Epitope-bearing peptides in accordance with the invention can be prepared synthetically, by recombinant DNA technology, or from natural sources such as whole viruses or tumors. Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides are synthetically conjugated to native molecules or particles; the peptides can also be conjugated to non-native molecules or particles.

[0076] The peptides in accordance with the invention can be a variety of lengths, and either in their neutral (uncharged) forms or in forms which are salts. The peptides in accordance with the invention are either free of modifications such as glycosylation, side chain oxidation, or phosphorylation; or they contain these modifications.

[0077] Desirably, the epitope-bearing peptide will be as small as possible while still maintaining relevant immunologic activity of the large peptide; of course it is particularly desirable with peptides from pathogenic organisms that the peptide be small in order to avoid pathogenic function. When possible, it may be desirable to optimize epitopes of the invention to a length of about 6 to about 25, preferably 14 to 15 amino acid residues for a class II molecule. Preferably, the peptides are commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to HLA class I or class II molecules on the cell surface. Nevertheless, the identification and preparation of peptides of other lengths can be carried out using the techniques described here such as the disclosures of primary anchor positions. It is to be appreciated that peptide epitopes in accordance with the invention can be present in peptides or proteins that are longer than the epitope itself. Moreover, multiepitopic peptides can comprise at least one epitope of the invention along with other epitope(s).

[0078] In particular, the invention provides motifs that are common to peptides bound by more than one HLA allele. By a combination of motif identification and MHC-peptide interaction studies, peptides useful for peptide vaccines have been identified.

[0079] Peptides comprising the epitopes from these antigens are synthesized and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, immunofluorescent staining and flow microfluorometry, peptide-dependent class II assembly assays. Those peptides that bind to the class II molecule are further evaluated for their ability to serve as targets for HTLs derived from infected or immunized individuals, as well as for their capacity to induce primary *in vitro* or *in vivo* HTL responses that can give rise to HTL populations capable of reacting with tumor cells as potential therapeutic agents.

[0080] The starting point, therefore, for the design of effective vaccines is to ensure that the vaccine will generate a large number of epitopes that can successfully be presented. It may be possible to administer the peptides representing the epitopes *per se*. Such administration is dependent on the presentation of "empty" HLA molecules displayed on the cells of the subject. In one approach to use of the immunogenic peptides *per se*, these peptides may be incubated with antigen-presenting cells from the subject to be treated *ex vivo* and the cells then returned to the subject.

[0081] Alternatively, the peptides can be generated *in situ* by administering a nucleic acid containing a nucleotide sequence encoding it. Means for providing such nucleic acid molecules are described in WO99/58658. Further, the immunogenic peptides can be administered as portions of a larger peptide molecule and cleaved to release the desired peptide. The larger peptide may contain extraneous amino acids, in general the fewer the better. Thus, peptides which contain such amino acids are typically 50 amino acids or less, more typically 30 amino acids or less, and more typically 20 amino acids or less. The precursor may also be a heteropolymer or homopolymer containing a multiplicity of different or same HTL epitopes. Of course, mixtures of peptides and nucleic acids which generate a variety of immunogenic peptides can also be employed. The design of the peptide vaccines, the nucleic acid molecules, or the hetero- or homo-polymers is dependent on the inclusion of the desired epitope.

[0082] In certain embodiments, it is preferred that peptides include an epitope that binds to an HLA-DR supertype allele. These motifs may be used to define T-cell

epitopes from any desired antigen, particularly those associated with human cancers for which the amino acid sequence of the potential antigen targets is known.

[0083] The peptides are thus useful in pharmaceutical compositions for both *in vivo* and *ex vivo* therapeutic and diagnostic applications.

[0084] Peptides comprising the supermotif sequences can be identified, as noted above, by screening potential antigenic sources. Useful peptides can also be identified by synthesizing peptides with systematic or random substitution of the variable residues in the supermotif, and testing them according to the assays provided. As demonstrated below, it is useful to refer to the sequences of the target HLA molecule, as well.

[0085] For epitope-based vaccines, the peptides of the present invention preferably comprise a supermotif and/or motif recognized by an HLA class II molecule having a wide distribution in the human population. The large degree of HLA polymorphism is an important factor to be taken into account with the epitope-based approach to vaccine development. To address this factor, epitope selection encompassing identification of peptides capable of binding at high or intermediate affinity to multiple HLA molecules is preferably utilized, most preferably these epitopes bind at high or intermediate affinity to two or more allele-specific HLA molecules.

[0086] HTL-inducing peptides of interest for vaccine compositions preferably include those that have an IC_{50} or binding affinity value for class II HLA molecules, 1000 nM or better (*i.e.*, the value is greater than or equal to 1000 nM). For example, peptide binding is assessed by testing the capacity of a candidate peptide to bind to a purified HLA molecule *in vitro*. Peptides exhibiting high or intermediate affinity are then considered for further analysis. Selected peptides are generally tested on other members of the supertype family. In preferred embodiments, peptides that exhibit cross-reactive binding are then used in cellular screening analyses or vaccines.

[0087] Definition of motifs that are predictive of binding to specific class II alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs and/or supermotifs.

[0088] The previous definition of motifs specific for different class II alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino

acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The capacity to bind MHC Class II molecules is measured in a variety of different ways.

[0089] The procedures used to identify peptides of the present invention generally follow the methods disclosed in Falk *et al.*, Nature 351:290 (1991). Briefly, the methods involve large-scale isolation of MHC class II molecules, typically by immunoprecipitation or affinity chromatography, from the appropriate cell or cell line. Examples of other methods for isolation of the desired MHC molecule equally well known to the artisan include ion exchange chromatography, lectin chromatography, size exclusion, high performance ligand chromatography, and a combination of all of the above techniques.

[0090] The peptides bound to the peptide binding groove of the isolated MHC molecules are eluted typically using acid treatment. Peptides can also be dissociated from class II molecules by a variety of standard denaturing means, such as heat, pH, detergents, salts, chaotropic agents, or a combination thereof.

[0091] Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

[0092] Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M. W., et al., Methods Enzymol. 91, 399 [1983]). Other methods suitable for sequencing include mass spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992)). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic sequence motif for each class I allele.

[0093] Next, peptides that test positive in the MHC class II binding assay are assayed for the ability of the peptides to induce specific HTL responses *in vitro*. For instance, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce HTL responses in responder cell populations. Antigen-presenting

cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., *J. Exp. Med.* 166:182 (1987); Boog, *Eur. J. Immunol.* 18:219 (1988)).

[0094] As disclosed herein, higher HLA binding affinity is correlated with greater immunogenicity. Greater immunogenicity can be manifested in several different ways. Immunogenicity can correspond to whether an immune response is elicited at all, and to the vigor of any particular response, as well as to the extent of a diverse population in which a response is elicited. For example, a peptide might elicit an immune response in a diverse array of the population, yet in no instance produce a vigorous response. In accordance with the principles disclosed herein, close to 90% of high binding peptides have been found to be immunogenic, as contrasted with about 50% of the peptides which bind with intermediate affinity. Moreover, higher binding affinity peptides lead to more vigorous immunogenic responses. As a result, less peptide is required to elicit a similar biological effect if a high affinity binding peptide is used. Thus, in preferred embodiments of the invention, high affinity binding epitopes are particularly useful. Nevertheless, improvements over the prior art are achieved with intermediate or high binding peptides.

[0095] After determining their binding affinity, additional confirmatory work can be performed to select, amongst these vaccine candidates, epitopes with preferred characteristics in terms of population coverage, antigenicity, and immunogenicity.

[0096] Thus, various strategies can be utilized to evaluate immunogenicity, including:

1) Evaluation of primary T cell cultures from normal individuals (*see, e.g.*, Wentworth, P. A. *et al.*, *Mol. Immunol.* 32:603, 1995; Celis, E. *et al.*, *Proc. Natl. Acad. Sci. USA* 91:2105, 1994; Tsai, V. *et al.*, *J. Immunol.* 158:1796, 1997; Kawashima, I. *et al.*, *Human Immunol.* 59:1, 1998); This procedure involves the stimulation of peripheral blood lymphocytes (PBL) from normal subjects with a test peptide in the presence of antigen presenting cells *in vitro* over a period of several weeks. T cells specific for the peptide become activated during this time and are detected.

2) Immunization of HLA transgenic mice (*see, e.g.*, Wentworth, P. A. *et al.*, *J. Immunol.* 26:97, 1996; Wentworth, P. A. *et al.*, *Int. Immunol.* 8:651, 1996; Alexander, J. *et al.*, *J. Immunol.* 159:4753, 1997); In this method, peptides in incomplete Freund's adjuvant are administered subcutaneously to HLA transgenic mice. Several weeks following immunization, splenocytes are removed and cultured *in vitro* in the

presence of test peptide for approximately one week. Peptide-specific T cells are detected.

3) Demonstration of recall T cell responses from patients who have been effectively vaccinated or who have a tumor; (*see, e.g.*, Rehermann, B. *et al.*, *J. Exp. Med.* 181:1047, 1995; Doolan, D. L. *et al.*, *Immunity* 7:97, 1997; Bertoni, R. *et al.*, *J. Clin. Invest.* 100:503, 1997; Threlkeld, S. C. *et al.*, *J. Immunol.* 159:1648, 1997; Diepolder, H. M. *et al.*, *J. Virol.* 71:6011, 1997; Tsang *et al.*, *J. Natl. Cancer Inst.* 87:982-990, 1995; Disis *et al.*, *J. Immunol.* 156:3151-3158, 1996). In applying this strategy, recall responses are detected by culturing PBL from patients with cancer who have generated an immune response "naturally", or from patients who were vaccinated with tumor antigen vaccines. PBL from subjects are cultured *in vitro* for 1-2 weeks in the presence of test peptide plus antigen presenting cells (APC) to allow activation of "memory" T cells, as compared to "naive" T cells. At the end of the culture period, T cell activity is detected.

[0097] An immunogenic peptide epitope of the invention may be included in a polyepitopic vaccine composition comprising additional peptide epitopes of the same antigen, antigens from the same source, and/or antigens from a different source. Moreover, class II epitopes can be included along with class I epitopes. Peptide epitopes from the same antigen may be adjacent epitopes that are contiguous in sequence or may be obtained from different regions of the protein.

[0098] An epitope present in the peptides of the invention can be cross-reactive or non-cross-reactive in its interactions with MHC alleles and alleles subtypes. Cross-reactive binding of an epitope (or peptide) permits an epitope to be bound by more than one HLA molecule. Such cross-reactivity is also known as degenerate binding. A non-cross-reactive epitope would be restricted to binding a particular MHC allele or allele subtype.

Motifs Indicative of Class II HTL Inducing Peptide Epitope

[0099] The primary anchor residues of the HLA class II supermotifs and motifs are delineated below.

HLA DR-1-4-7 supermotif

[0100] Motifs have also been identified for peptides that bind to three common HLA class II allele-specific HLA molecules: HLA DRB1*0401, DRB1*0101, and DRB1*0701 (*see, e.g.*, the review by Southwood *et al. J. Immunology* 160:3363-3373,1998). Collectively, the common residues from these motifs delineate the HLA DR-1-4-7 supermotif. Peptides that bind to these DR molecules carry a supermotif characterized by a large aromatic or hydrophobic residue (Y, F, W, L, I, V, or M) as a primary anchor residue in position 1, and a small, non-charged residue (S, T, C, A, P, V, I, L, or M) as a primary anchor residue in position 6 of a 9-mer core region. Allele-specific secondary effects and secondary anchors for each of these HLA types have also been identified (Southwood *et al., supra*). Peptide binding to HLA-DRB1*0401, DRB1*0101, and/or DRB1*0701 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

[0101] Two alternative motifs (*i.e.*, submotifs) characterize peptide epitopes that bind to HLA-DR3 molecules (*see, e.g.*, Geluk *et al.*, *J. Immunol.* 152:5742, 1994). In the first motif (submotif DR3A) a large, hydrophobic residue (L, I, V, M, F, or Y) is present in anchor position 1 of a 9-mer core, and D is present as an anchor at position 4, towards the carboxyl terminus of the epitope. As in other class II motifs, core position 1 may or may not occupy the peptide N-terminal position.

[0102] The alternative DR3 submotif provides for lack of the large, hydrophobic residue at anchor position 1, and/or lack of the negatively charged or amide-like anchor residue at position 4, by the presence of a positive charge at position 6 towards the carboxyl terminus of the epitope. Thus, for the alternative allele-specific DR3 motif (submotif DR3B): L, I, V, M, F, Y, A, or Y is present at anchor position 1; D, N, Q, E, S, or T is present at anchor position 4; and K, R, or H is present at anchor position 6. Peptide binding to HLA-DR3 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the motif.

[0103] As with HLA class I binding peptides, motifs have also been defined for HLA class II-binding peptides. Several studies have identified an important role for an aromatic or hydrophobic residue (I, L, M, V, F, W, or Y) at position 1 of a 9-mer core region, typically nested within a longer peptide sequence, in the binding of peptide

ligands to several HLA-class II alleles (Hammer et al. *Cell* **74**:197, (1993); Sette et al. *J. Immunol.* **151**:3163-70 (1993); O'Sullivan et al. *J. Immunol.* **147**:2663 (1991); and Southwood et al. *J. Immunol.* **160**:3363-73 (1998)). A strong role has also been demonstrated for the residue in position 6 of the 9-mer core, where short and/or hydrophobic residues (S, T, C, A, P, V, I, L, or M) are preferred. This position 1-position 6 motif has been described as a DR-supermotif (Southwood et al. *J. Immunol.* **160**:3363-3373 (1998)) and has been shown to efficiently identify peptides capable of binding a large set of common HLA-class II alleles.

[0104] Peptides binding to class II molecules may also be analyzed with respect to the identification of secondary preferred or deleterious residues. For example, to derive a more detailed DRB1*0401 motif to define secondary residues influencing peptide binding, we employed a strategy similar to that performed with class I peptides. For each peptide analyzed, nine-residue-long core regions were aligned on the basis of the primary class II positions P1 and P6 anchors. Then, the average binding affinity of a peptide carrying a particular residue was calculated for each position, relative to the remainder of the group. Following this method, values showing average relative binding were compiled. These values also present a map of the positive or negative effect of each of the 20 naturally occurring amino acids in DRB1*0401 binding capacity when occupying a particular position relative to the P1-P6 class II motif positions.

[0105] Variations in average relative binding of greater than or equal to fourfold or less than or equal to 0.25 were arbitrarily considered significant and indicative of secondary effects of a given residue on HLA-peptide interactions. Most secondary effects were associated with P4, P7, and P9. These positions correspond to secondary anchors engaging shallow pockets on the DR molecule. Similar studies defining secondary residues were also performed for DRB1*0101 and DRB1*0701. The definitions of secondary residues of motifs for DR1, DR4, and DR7 are shown in TABLE 139.

[0106] Upon definition of allele-specific secondary effects and secondary anchors, allele-specific algorithms were derived and utilized to identify peptides binding DRB1*0101, DRB1*0401, and DRB*0701. Further experiments, identified a large set of HLA class II molecules, which includes at least the DRB1*0101, DRB1*0401, and DRB*0701, DRB1*1501, DRB1*0901 and DRB1*1302 allelic products

recognizing the DR supermotif, and is characterized by largely overlapping peptide binding repertoires.

[0107] The data presented above confirm that several common HLA class II types are characterized by largely overlapping peptide binding repertoires. On this basis, in analogy to the case of HLA class I molecules, HLA class II molecules can be grouped in a HLA class II supertype, defined and characterized by similar, or largely overlapping (albeit not identical) peptide binding specificities.

[0108] The peptides present in the invention can be identified by any suitable method. For example, peptides are conveniently identified using the algorithms of the invention described in the co-pending U.S. Patent 7,462,354. These algorithms are mathematical procedures that produce a score which enables the selection of immunogenic peptides. Typically one uses the algorithmic score with a binding threshold to enable selection of peptides that have a high probability of binding at a certain affinity and will in turn be immunogenic. The algorithm are based upon either the effects on MHC binding of a particular amino acid at a particular position of a peptide or the effects on binding MHC of a particular substitution in a motif containing peptide.

[0109] Peptide sequences characterized in molecular binding assays and capture assays have been and can be identified utilizing various technologies. Motif-positive sequences are identified using a customized application created at Epimmune. Sequences are also identified utilizing matrix-based algorithms, and have been used in conjunction with a "power" module that generates a predicted 50% inhibitory concentration (PIC) value. These latter methods are operational on Epimmune's HTML-based Epitope Information System (EIS) database. All of the described methods are viable options in peptide sequence selection for IC_{50} determination using binding assays.

[0110] The capacity to bind MHC molecules is measured in a variety of different ways. One means is a MHC binding assay as described in the related applications, noted above. Other alternatives described in the literature include inhibition of antigen presentation (Sette, *et al., J. Immunol.* 141:3893 (1991), *in vitro* assembly assays (Townsend, *et al.*, Cell 62:285 (1990), and FACS based assays using mutated cells, such as RMA.S (Melief, *et al., Eur. J. Immunol.* 21:2963 (1991)).

[0111] Capture Assay: Unlike the HPLC-based molecular binding assay, noted above, the high throughput screening ("HTS") Capture assay does not utilize a size-exclusion silica column for separation of bound from unbound radioactive marker. Instead, wells of an opaque white 96-well Optiplate (Packard) are coated with 3μg (100μl @ 30μg/ml) of HLA-specific antibody (Ab) that "capture" complexes of radiolabeled MHC and unlabeled peptide transferred from the molecular binding assay plate in 100μl of 0.05% NP40/PBS. After a 3-hour incubation period, the supernatant is decanted and scintillation fluid (Microscint 20) added. Captured complexes are then measured on a microplate scintillation and luminescence counter (TopCount NXTTM; Packard).

[0112] Additional assays for determining binding are described in detail, *i.e.*, in PCT publications WO 94/20127 and WO 94/03205. Binding data results are often expressed in terms of IC_{50} value. IC_{50} is the concentration of peptide in a binding assay at which 50% inhibition of binding of a reference peptide occurs. Given the conditions in which the assays are preformed (*i.e.*, limiting MHC proteins and labeled peptide concentrations), these values approximate K_D values. It should be noted that IC_{50} values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (*i.e.*, MHC preparation, *etc.*). For example, excessive concentrations of MHC molecules will increase the apparent measured IC_{50} of a given ligand. Alternatively, binding is expressed relative to a reference peptide. Although as a particular assay becomes more, or less, sensitive, the IC_{50}'s of the peptides tested may change somewhat, the binding relative to the reference peptide will not significantly change. For example, in an assay preformed under conditions such that the IC_{50} of the reference peptide increases 10-fold, the IC_{50} values of the test peptides will also increase approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder is generally based on its IC_{50}, relative to the IC_{50} of a standard peptide.

[0113] The peptides of the invention may also comprise isosteres of two or more residues in the MHC-binding peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the

α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. *See, generally*, Spatola, *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. VII (Weinstein ed., 1983).

[0114] Modifications of peptides with various amino acid mimetics or unnatural amino acids are particularly useful in increasing the stability of the peptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al., Eur. J. Drug Metab. Pharmacokin. 11:291-302 (1986). Half life of the peptides of the present invention is conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows. Pooled human serum (Type AB, non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI tissue culture media and used to test peptide stability. At predetermined time intervals a small amount of reaction solution is removed and added to either 6% aqueous trichloracetic acid or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

[0115] Such analogs may also possess improved shelf-life or manufacturing properties. More specifically, non-critical amino acids need not be limited to those naturally occurring in proteins, such as L-α-amino acids, or their D-isomers, but may include non-natural amino acids as well, such as amino acids mimetics, *e.g.* D- or L-naphylalanine; D- or L- phenylglycine; D- or L-2-thieneylalanine; D- or L-1, -2, 3-, or 4- pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-ρ-fluorophenylalanine; D- or L- ρ-biphenylphenylalanine; D- or L- ρ-methoxybiphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylalanines, where the alkyl group can be a substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a nonnatural amino acid include, *e.g.*, thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

[0116] Another embodiment for generating effective peptide analogs involves the substitution of residues that have an adverse impact on peptide stability or solubility in, *e.g.*, a liquid environment. This substitution may occur at any position of the peptide epitope. Analogs of the present invention may include peptides containing substitutions to modify the physical property (e.g., stability or solubility) of the resulting peptide. For example, a cysteine (C) can be substituted out in favor of α-amino butyric acid. Duc to its chemical nature, cysteine has the propensity to form disulfide bridges and sufficiently alter the peptide structurally so as to reduce binding capacity. Substituting α-amino butyric acid for C not only alleviates this problem, but actually improves binding and crossbinding capability in certain instances (*see*, *e.g.*, the review by Sette *et al.*, In: Persistent Viral Infections, Eds. R. Ahmed and I. Chen, John Wiley & Sons, England, 1999). Substitution of cysteine with α-amino butyric acid may occur at any residue of a peptide epitope, *i.e.* at either anchor or non-anchor positions.

[0117] The binding activity, particularly modification of binding affinity or cross-reactivity among HLA supertype family members, of peptides of the invention can also be altered using analoging, which is described in co-pending U.S. application number 09/226,775 filed 1/6/99. In brief, the analoging strategy utilizes the motifs or supermotifs that correlate with binding to certain HLA molecules. Analog peptides can be created by substituting amino acid residues at primary anchor, secondary anchor, or at primary and secondary anchor positions. Generally, analogs are made for pcptidcs that already bear a motif or supermotif. For a number of the motifs or supermotifs in accordance with thc invention, residues are defined which are deleterious to binding to allclc-spccific HLA molecules or members of HLA supertypcs that bind the respective motif or supermotif (*see*, e.g., Rupert *et al. Cell* 74:929, 1993; Sidney, J. *et al.*, *Hu. Immunol.* 45:79, 1996; and Sidney *et al.*; Sidney, *et al., J. Immunol.* 154:247, 1995). Accordingly, removal of such residues that are detrimental to binding can be performed in accordance with the present invention. For example, in the case of the A3 supertype, when all peptides that have such deleterious residues are removed from the population of peptides used in the analysis, the incidence of cross-reactivity increased from 22% to 37% (*see*, *e.g.*, Sidney, J. *et al.*, *Hu. Immunol.* 45:79, 1996).

[0118] Thus, one strategy to improve the cross-reactivity of peptides within a given supermotif is simply to delete one or more of the deleterious residues present within a peptide and substitute a small "neutral" residue such as Ala (that may not influence T cell recognition of the peptide). An enhanced likelihood of cross-reactivity is expected if, together with elimination of detrimental residues within a peptide, "preferred" residues associated with high affinity binding to an allele-specific HLA molecule or to multiple HLA molecules within a superfamily are inserted.

[0119] In some embodiments, a T helper peptide can used in addition to one of the peptides of the invention. One type of T helper peptide is one that is recognized by T helper cells in the majority of the population. This can be accomplished by selecting amino acid sequences that bind to many, most, or all of the MHC class II molecules. These are known as "loosely MHC-restricted" T helper sequences. Examples of amino acid sequences that are loosely MHC-restricted include sequences from antigens such as Tetanus toxin at positions 830-843 (QYIKANSKFIGITE (SEQ ID NO:1)), *Plasmodium falciparum* circumsporozoite (CS) protein at positions 378-398 (DIEKKIAKMEKASSVFNVVNS (SEQ ID NO:2)), and Streptococcus 18 kD protein at positions 1-16 (YGAVDSILGGVATYGAA (SEQ ID NO:3)).

[0120] Alternatively, it is possible to prepare synthetic peptides capable of stimulating T helper lymphocytes, in a loosely MHC-restricted fashion, using amino acid sequences not found in nature (*see, e.g.*, PCT publication WO 95/07707). These synthetic compounds, called Pan-DR-binding epitopes or PADRE® molecules (Epimmune, San Diego, CA), are designed on the basis of their binding activity to most HLA-DR (human MHC class II) molecules (*see, e.g.*, USSN 08/121,101 (now abandoned) and related USSN 08/305,871 (now U.S. Patent Number 5,736,142)). For instance, a pan-DR-binding epitope peptide having the formula: aKXVWANTLKAAa (SEQ ID NO:4), where X is either cyclohexylalanine, phenylalanine, or tyrosine, and "a" is either D-alanine or L-alanine, has been found to bind to most HLA-DR alleles, and to stimulate the response of T helper lymphocytes from most individuals, regardless of their HLA type.

[0121] Particularly preferred immunogenic peptides and/or T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically

selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the HTL peptide may be linked to the T helper peptide without a spacer.

[0122] The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the HTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated. The T helper peptides used in the invention can be modified in the same manner as HTL peptides. For instance, they may be modified to include D-amino acids or be conjugated to other molecules such as lipids, proteins, sugars and the like. Exemplary T helper peptides include tetanus toxoid 830-843, influenza 307-319, malaria circumsporozoite 382-398 and 378-389.

[0123] In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes HTL and CTL. Lipids have been identified as agents capable of priming HTL and CTL *in vivo* against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide. Also in a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of a class I restricted peptide having T cell determinants, such as those peptides described herein as well as other peptides which have been identified as having such determinants.

[0124] As another example of lipid priming of HTL and CTL responses, E. coli lipoproteins, such as tripalmitoyl-S-glycerylcysteinlyseryl-serine (P_3CSS) can be used to prime virus specific HTL CTL when covalently attached to an appropriate peptide.

See, Deres *et al*, Nature 342:561-564 (1989). Peptides of the invention can be coupled to P_3CSS, for example, and the lipopeptide administered to an individual to specifically prime a HTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with P_3CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell- mediated responses to infection.

[0125] In addition, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support, or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. Modification at the C terminus in some cases may alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH2 acylation, e.g., by alkanoyl (C_1-C_{20}) or thioglycolyl acetylation, terminal-carboxyl amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

[0126] The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), *supra*.

[0127] Another aspect of the present invention is directed to vaccines which comprise an immunogenically effective amount of one or more peptides as described herein. Peptides may be introduced into a host using a variety of delivery vehicles known to those of skill in the art including PLG microspheres with entrapped peptides and virus-like particles. Furthermore, epitopes may be introduced as multiple antigen peptides (MAPs) (see e.g., Mora and Tarn, *J. Immunol.* 161:3616-23 (1998)), or as immunostimulating complexes (ISCOMS) (see e.g., *Hu et al. Clin. Exp. Immunol.* 113:235-43 (1998)) as known in the art.

[0128] Vaccines that contain an immunogenically effective amount of one or more peptides as described herein are a further embodiment of the invention. The vaccines of the invention can be used both as a prevantative or therapeutic. Once appropriately immunogenic epitopes have been defined, they can be delivered by various means, herein referred to as "vaccine" compositions. Such vaccine compositions can include, for example, lipopeptides *(e.g.,* Vitiello, A. *et al.,* J: *Clin. Invest.* 95:341, 1995), peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres *(see, e.g.,* Eldridge, *et al., Molec. Immunol.* 28:287-294, 1991: Alonso *et al., Vaccine* 12:299-306, 1994; Jones *et al., Vaccine* 13:675-681, 1995), peptide compositions contained in immunc stimulating complexes (ISCOMS) *(see, e.g.,* Takahashi *et al., Nature* 344:873-875, 1990; Hu *et al., Clin Exp Immunol.* 113:235-24: 1998), multiple antigen pcptide systems (MAPs) *(see e.g.,* Tam, J. P., *Proc. Nati. Acaa Sci. U.S.A.* 85:5409-5413, 1988; Tam, *J.P.,J Immunol. Methods* 196:17-32, 1996), vir delivery vectors (Perkus, M. E. *et al.,* In: *Concepts in vaccine development,* Kaufmann H. E., ed., p. 379, 1996; Chakrabarti, S. *et al., Nature* 320:535,1986; Hu, S. L. *et al., Nature* 320:537, 1986; Kieny, M.-P. *et al., AIDS Bio/Technology* 4:790, 1986; Top, F. *et al., J Infect. Dis.* 124:148, 1971; Chanda, P. K. *et al., Virology* 175:535, 1990), particles of viral or synthetic origin *(e.g.,* Kofler, N. *et al., J Immunol. Methods.* 192:2~ 1996; Eldridgc, J. H. *et al., Sem. Bematol.* 30:16, 1993; Fa10, L. D., Jr. *et al., Nature Med.* 7:649, 1995), adjuvants (Warren, H. S., Vogel, F. R., and Chedid, L. A. *Annu. Re Immunol.* 4:369, 1986; Gupta, R. K. *et al., Vaccine* 11:293, 1993), 1iposomes (Reddy, R *et al., J. Immunol.* 148:1585, 1992; Rock, K. L., *Immunol. Today* 17:131, 1996), or, naked or particle absorbed cDNA (Ulmer, J. B. *et al., Science* 259:1745, 1993; Robinsol H. L., Hunt, L. A., and Webster, R. G., *Vaccine* 11:957, 1993; Shiver, J. W. *et al.,* In: *Concepts in vaccine development,* Kaufmann, S. H. E., ed., p. 423, 1996; Cease, K. B., and Berzofsky, J. A., *Annu. Rev. Immunol.* 12:923, 1994 and Eldridge, J. H. *et al., Sem. Hematol.* 30:16, 1993). Toxin-targeted delivery technologies, also known as receptor mediated targeting, such as those of Avant Immunotherapeutics, Inc. (Needham, Massachusetts) may also be used.

[0129] Vaccine compositions of the invention include nucleic acid-mediated modalities. DNA or RNA encoding one or more of the peptides of the invention can also be administered to a patient. This approach is described, for instance, in Wolff *et. al., Science* 247: 1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466;

5,804,566; 5,739,118; 5,736,524; 5,679,647; WO 98/04720; and in more detail below. Examples of DNA-based delivery technologies include "naked DNA", facilitated (bupivicaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or pressure-mediated delivery *(see, e.g.,* U.S. Patent No. 5,922,687).

[0130] For therapeutic or prophylactic immunization purposes, the peptides of the invention can be expression vectors include attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus, for example, as a vector to express nucleotide sequences that encode the pep tides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL and/or HTL response. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.,* U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover *et al., Nature* 351:456-460 (1991). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, *e.g.* adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella typhi* vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art from the description herein.

[0131] Furthermore, vaccines in accordance with the invention can encompass one or more of the peptides of the invention. Accordingly, a peptide can be present in a vaccine individually. Alternatively, the peptide can be individually linked to its own carrier; alternatively, the peptide can exist as a homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides. Polymers have the advantage of increased immunological reaction and, where different peptide epitopes are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the pathogenic organism or tumor-related peptide targeted for an immune response. The composition may be a naturally occurring region of an antigen or may be prepared, *e.g.,* recombinantly or by chemical synthesis.

[0132] Carriers that can be used with vaccines of the invention are well known in the art, and include, *e.g.,* thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza,

hepatitis B virus core protein, and the like. The vaccines can contain a physiologically tolerable (*i.e.*, acceptable) diluent such as water, or saline, preferably phosphate buffered saline. The vaccines also typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are examples of materials well known in the art. Additionally, CTL responses can be primed by conjugating peptides of the invention to lipids, such as tripalmitoyl-S-glycerylcysteinlyseryl- serine (P3CSS).

[0133] Upon immunization with a peptide composition in accordance with the invention, via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, the immune system of the host responds to the vaccine by producing large amounts of HTLs and/or CTLs specific for the desired antigen. Consequently, the host becomes at least partially immune to later infection, or at least partially resistant to developing an ongoing chronic infection, or derives at least some therapeutic benefit when the antigen was tumor-associated.

[0134] For therapeutic or immunization purposes, the peptides of the invention can also be expressed by vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover, *et al. Nature* 351:456-60 (1991). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, *e.g., Salmonella typhi* vectors, retroviral vectors, adenoviral or adeno-associated viral vectors, and the like will be apparent to those skilled in the art from the description herein.

[0135] Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982) (also 1989). Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the

appropriate T cell epitope. For example, a coding sequence encoding a peptide of the invention can be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. Expression constructs, *i.e.*, minigenes are described in greater detail in the sections below. Such methodologies are also used to present at least one peptide of the invention along with a substance which is not a peptide of the invention.

[0136] As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, using the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), with modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

[0137] The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent cancer.

[0138] In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective HTL response to the tumor antigen and to cure or at least partially arrest symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose" or "unit dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and

general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0 μg to about 5000 μg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μg to about 1000 μg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. In alternative embodiments, generally for humans the dose range for the initial immunization (that is for therapeutic or prophylactic administration) is from about 1.0 μg to about 20,000 μg of peptide for a 70 kg patient, preferably, 100 μg -, 150 μg -, 200 μg -, 250 μg -, 300 μg -, 400 μg -, or 500 μg -20,000 μg, followed by boosting dosages in the same dose range pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific HTL activity in the patient's blood. In embodiments where recombinant nucleic acid administration is used, the administered material is titrated to achieve the appropriate therapeutic response.

[0139] It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

[0140] For therapeutic use, administration should begin at the first sign of tumors or shortly after diagnosis. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter.

[0141] Treatment of an affected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developingcancer the compositions are particularly useful in methods for preventing the evolution of cancer.

[0142] The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides

dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

[0143] A pharmaceutical composition of the invention may comprise one or more T cell stimulatory peptides of the invention. For example, a pharmaceutical composition may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more T cell stimulatory peptides of the invention. Moreover, a pharmaceutical composition of the invention may comprise one or more T cell stimulatory peptides of the invention in combination with one or more other T cell stimulatory peptides. The concentration of each unique T cell stimulatory peptide of the invention in the pharmaceutical formulations can vary widely, *e.g.*, from less than about 0.001%, about 0.002%, about 0.003%, about 0.004%, about 0.005%, about 0.006%, 0.007%, 0.008%, 0.009%, about 0.01%, about 0.02%, about 0.025%, about 0.03%, about 0.04%, about 0.05%, about 0.06%, about 0.07%, about 0.08%, about 0.09%, about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1%, about 1.1%, about 1.2%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 20%, to about 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. In a preferred embodiment, the concentration of each unique T cell stimulatory peptide of the invention in the pharmaceutical formulations is about 0.001%, about 0.002%, about 0.003%, about 0.004%, about 0.005%, about 0.006%, 0.007%, 0.008%, 0.009%, about 0.01%, about 0.02%, about 0.025%, about 0.03%, about 0.04%, about 0.05%, about 0.06%, about

0.07%, about 0.08%, about 0.09%, about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1% by weight. In a more preferred embodiment, the concentration of each unique T cell stimulatory peptide of the invention in the pharmaceutical formulations is about 0.01%, about 0.02%, about 0.025%, about 0.03%, about 0.04%, about 0.05%, about 0.06%, about 0.07%, about 0.08%, about 0.09%, about 0.1% by weight.

[0144] The concentration of HTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. A human unit dose form of the peptide composition is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable carrier, preferably an aqueous carrier, and is administered in a volume of fluid that is known by those of skill in the art to be used for administration of such compositions to humans.

[0145] The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng.

9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

[0146] For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

[0147] For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

[0148] For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

[0149] In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to

induce antibodies and/or CTLs that react with different antigenic determinants of tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of HTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

[0150] The peptides of the present invention and pharmaceutical and vaccine compositions of the invention are useful for administration to mammals, particularly humans, to treat and/or prevent cancer. Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk of cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 μg to about 5000 μg per 70 kilogram patient, more commonly from about 10 μg to about 500 μg mg per 70 kg of body weight.

[0151] As noted herein, the peptides of the invention induce HTL immune responses when contacted with a HTL specific to an epitope comprised by the peptide. The manner in which the peptide is contacted with the HTL is not critical to the invention. For instance, the peptide can be contacted with the HTL either *in vivo* or *in vitro*. If the contacting occurs *in vivo*, the peptide itself can be administered to the patient or other vehicles, *e.g.*, DNA vectors encoding one or more peptide, viral vectors encoding the peptide(s), liposomes and the like, can be used, as described herein.

[0152] For therapeutic or immunization purposes, nucleic acids encoding one or more of the peptides of the invention can also be administered to the patient. A number of

methods are conveniently used to deliver the nucleic acids to the patient. For instance, the nucleic acid can be delivered directly, as "naked DNA". This approach is described, for instance, in Wolff *et. al., Science* 247: 1465-68 (1990) as well as U.S. Patent Nos. 5,580,859 and 5,589,466. The nucleic acids can also be administered using ballistic delivery as described, for instance, in U.S. Patent No. 5,204,253. Particles comprised solely of DNA can be administered. Alternatively, DNA can be adhered to particles, such as gold particles.

[0153] The nucleic acids can also be delivered complexed to cationic compounds, such as cationic lipids. Lipid-mediated gene delivery methods are described, for instance, in WO 96/18372; WO 93/24640; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7): 682-691; Rose U.S. Pat No. 5,279,833; WO 91/06309; and Felgner *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 7413-14.

[0154] Nucleic acids encoding one or more of the peptides of the invention can also be administered to the patient. This approach is described, for instance, in Wolff, *et. al.*, Science, 247:1465-68 (1990) as well as U.S. Patent Nos. 5,580,859 and 5,589,466.

[0155] A preferred means of administering nucleic acids encoding the peptides of the invention uses minigene constructs encoding multiple epitopes of the invention. To create a DNA sequence encoding the selected HTL epitopes (minigene) for expression in human cells, the amino acid sequences of the epitopes are reverse translated. A human codon usage table is used to guide the codon choice for each amino acid. These epitope-encoding DNA sequences are directly adjoined, creating a continuous polypeptide sequence. To optimize expression and/or immunogenicity, additional elements can be incorporated into the minigene design. Examples of amino acid sequence that could be reverse translated and included in the minigene sequence include: a leader (signal) sequence, and an endoplasmic reticulum retention signal. In addition, MHC presentation of HTL epitopes may be improved by including synthetic (e.g. poly-alanine) or naturally-occurring flanking sequences adjacent to the HTL epitopes.

[0156] The minigene sequence is converted to DNA by assembling oligonucleotides that encode the plus and minus strands of the minigene. Overlapping oligonucleotides (30-100 bases long) are synthesized, phosphorylated, purified and annealed under appropriate conditions using well known techniques. The ends of the oligonucleotides

are joined using T4 DNA ligase. This synthetic minigene, encoding the HTL epitope polypeptidc, can then cloned into a desired expression vector.

[0157] Standard regulatory sequences well known to those of skill in the art are included in the vector to ensure expression in the target cells. Several vector elements are required: a promoter with a down-stream cloning site for minigene insertion; a polyadenylation signal for efficient transcription termination; an *E. coli* origin of replication; and an *E. coli* selectable marker (e.g. ampicillin or kanamycin resistance). Numerous promotcrs can be used for this purpose, *e.g.*, the human cytomegalovirus (hCMV) promoter. Scc, U.S. Patent Nos. 5,580,859 and 5,589,466 for other suitable promoter sequences.

[0158] Additional vcctor modifications may be desired to optimize minigene expression and immunogenicity. In some cases, introns are required for efficient gene expression, and one or morc synthetic or naturally-occurring introns could be incorporated into the transcribed region of the minigene. The inclusion of mRNA stabilization sequenccs can also be considered for increasing minigene expression. It has recently bccn proposed that immunostimulatory sequences (ISSs or CpGs) play a role in the immunogenicity of DNA vaccines. These sequences could be included in the vector, outside thc minigene coding sequence, if found to enhance immunogenicity.

[0159] In some embodiments, a bicistronic expression vector, to allow production of the minigene-encoded epitopes and a second protein included to enhance or decrease immunogenicity can be used. Examples of proteins or polypeptides that could beneficially enhancc the immune response if co-expressed include cytokines (e.g., IL2, IL12, GM-CSF), cytokine-inducing molecules (e.g., LeIF) or costimulatory molecules. Helper (HTL) epitopes could be joined to intracellular targeting signals and exprcssed separately from the CTL epitopes. This would allow direction of the HTL epitopes to a cell compartment different than the CTL epitopes. If required, this could facilitate more efficient entry of HTL epitopes into the MHC class II pathway, thereby improving CTL induction. In contrast to CTL induction, specifically dccreasing the immune response by co-expression of immunosuppressive molecules (e.g. TGF-β) may be beneficial in certain diseases.

[0160] Oncc an expression vector is selected, the minigene is cloned into the polylinker region downstream of the promoter. This plasmid is transformed into an

appropriate *E. coli* strain, and DNA is prepared using standard techniques. The orientation and DNA sequence of the minigene, as well as all other elements included in the vector, are confirmed using restriction mapping and DNA sequence analysis. Bacterial cells harboring the correct plasmid can bc stored as a master cell bank and a working cell bank.

[0161] Therapeutic quantities of plasmid DNA are produced by fermentation in E. coli, followed by purification. Aliquots from the working cell bank are used to inoculate fermentation medium (such as Terrific Broth), and grown to saturation in shaker flasks or a bioreactor according to wcll known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins supplied by Quiagcn. If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods.

[0162] Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffer saline (PBS). A variety of methods have been described, and new techniques may become available. As noted above, nucleic acids are conveniently formulated with cationic lipids. In addition, glycolipids, fusogenic liposomes, peptides and compounds referred to collectively as protective, interactive, non-condensing (PINC) could also bc complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types.

[0163] The nucleic acids can also be administered using ballistic delivery as described, for instance, in U.S. Patcnt No. 5,204,253. Particles comprised solely of DNA can be administered. Alternatively, DNA can be adhered to particles, such as gold particles.

[0164] *In vivo* immunogenicity is a second approach for functional testing of minigene DNA formulations. Transgenic mice expressing appropriate human MHC molecules are immunized with the DNA product. The dosc and route of administration are formulation dependent (e.g. IM for DNA in PBS, IP for lipid-complexed DNA). Twenty-one days aftcr immunization, splenocytes are harvested and restimulated for 1 week in the presence of peptides encoding each epitope being tested.

[0165] An embodiment of a vaccine composition in accordance with the invention comprises *ex vivo* administration of a cocktail of epitope-bearing peptides to PBMC, or isolated DC therefrom, from the patient's blood. After pulsing the DC with peptides and prior to reinfusion into patients, the DC are washed to remove unbound peptides. In this embodiment, a vaccine comprises peptide-pulsed DCs which present the pulsed peptide epitopes in HLA molecules on their surfaces.

[0166] Dendritic cells can also be transfected, *e.g.,* with a minigene comprising nucleic acid sequences encoding the epitopes in accordance with the invention, in order to elicit immune responses. Vaccine compositions can be created *in vitro,* following dendritic cell mobilization and harvesting, whereby loading of dendritic cells occurs *in vitro.*

[0167] Transgenic animals of appropriate haplotypes may additionally provide a useful tool in optimizing the *in vivo* immunogenicity of minigene DNA. In addition, animals such as monkeys having conserved HLA molecules with cross reactivity to CTL epitopes recognized by human MHC molecules can be used to determine human immunogenicity of CTL epitopes (Bertoni, *et al., J. Immunol.* 161:4447-4455 (1998)).

[0168] Such *in vivo* studies are required to address the variables crucial for vaccine development, which are not easily evaluated by *in vitro* assays, such as route of administration, vaccine formulation, tissue biodistribution, and involvement of primary and secondary lymphoid organs. Because of their simplicity and flexibility, HLA transgenic mice represent an attractive alternative, at least for initial vaccine development studies, compared to more cumbersome and expensive studies in higher animal species, such as nonhuman primates.

[0169] Antigenic peptides are used to elicit a HTL response ex *vivo,* as well. The resulting HTL cells, can be used to treat tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a therapeutic vaccine peptide or nucleic acid in accordance with the invention. *Ex vivo* HTL responses to a particular antigen are induced by incubating in tissue culture the patient's (HTLp), or genetically compatible, HTL precursor cells together with a source of antigen-presenting cells (APC), such as dendritic cells, and the appropriate immunogenic peptide. After an appropriate incubation time (typically about 7-28 days (1-4 weeks)), in which the precursor cells are activated and matured and expanded into effector cells, the cells are infused back into the patient, where they will destroy their specific

target cell (an infected cell or a tumor cell). Trans fected dendritic cells may also be used as antigen presenting cells. In order to optimize the *in vitro* conditions for the generation of specific helper T cells, the culture of stimulator cells is maintained in an appropriate serum-free medium.

[0170] The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic infection.

[0171] For example, a peptide of the invention may be used in a tetramer staining assay to assess peripheral blood mononuclear cells for the presence of antigen-specific CTLs following exposure to a pathogen or immunogen. The HLA-tetrameric complex is used to directly visualize antigen-specific CTLs (see, *e.g.*, Ogg, *et al. Science* 279:2103-2106, 1998; and Altman, *et al. Science* 174:94-96, 1996) and determine the frequency of the antigen-specific CTL population in a sample of peripheral blood mononuclear cells. A tetramer reagent using a peptide of the invention may be generated as follows: A peptide that binds to an allele-specific HLA molecule or supertype molecule is refolded in the presence of the corresponding HLA heavy chain and β_2-microglobulin to generate a trimolecular complex. The complex is biotinylated at the carboxyl terminal end of the heavy chain at a site that was previously engineered into the protein. Tetramer formation is then induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to stain antigen-specific cells. The cells may then be identified, for example, by flow cytometry. Such an analysis may be used for diagnostic or prognostic purposes. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic infection.

[0172] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this

invention that certain changes and modifications may be made thereto without departing from the scope of the appended claims.

EXAMPLES

Materials and Methods

[0173] The following materials and methods apply generally to all the examples disclosed herein. Specific materials and methods are disclosed in each example, as necessary.

[0174] Reagents: Anti-IFN-γ and biotinylated anti- IFN-γ were obtained from Mabtech (Sweden). Phorbol myristate acetate (PMA), human serum albumin (HSA), polyclonal human IgG, tetanus toxin (TT), and ionomycin were from Sigma (St. Louis, MO, USA). Goat anti-human horseradish peroxidase (HRP)-conjugated antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Hank's balanced salts solution (HBSS), RPMI-1640 and phosphate-buffered saline were from Cellgro (Herndon, VA, USA). Ficoll-Paque was from Amersham Biosciences (Uppsala, Sweden). All peptides were synthesized by either the Mayo Clinic Protein Chemistry and Proteomics Core or by Epimmune, Inc. (San Diego, CA) and purified to >95% homogeneity by reverse-phase HPLC as previously described (Dzuris JL, Sidney J, Appella E, Chesnut RW, Watkins DI, Sette A. Conserved MHC class I peptide binding motif between humans and rhesus macaques. J Immunol 2000;164: 283-91). Purity of peptides was determined with reverse-phase HPLC and amino acid analysis, sequencing, and/or mass spectrometry. Lyophilized peptides were resuspended at 20 mg/ml in 100% DMSO and then diluted to required concentrations in PBS.

[0175] Epitope Prediction: The prediction program used, PIC (Predicted IC50), is a modified linear coefficient, or matrix-based method for predicting peptides with HLA-DR binding capacity. PIC is predicated on the assumption that each residue along a peptide molecule can independently contribute to binding affinity (Sette A, et al. Proc Natl Acad Sci U S A 1989;86: 3296-300; Sette A, et al. J Immunol 1989;142: 35-40). The algorithm yields a predicted IC50 value (designated as PIC) for the corresponding input sequence. Lower PIC values indicate a higher probability of

binding to HLA. The program analyzes 15 amino acid long scquences offset by 3 residues encompassing the entire protein.

[0176] Peripheral Blood Mononuclear Cell Preparation (PBMC): PBMC were isolated from blood as described (Disis ML, et al. Clin Cancer Res 1999;5: 1289-97), and cryopreserved in liquid nitrogen (20 x 106/ml cells) in freezing media (RPMI with 12.5% HSA, penicillin, streptomycin and 2 mM glutamine) (Disis ML *et al.* J Immunol Methods 2005.).

[0177] HLA-DR purification. Fifteen distinct HLA-DR molecules were used in quantitative assays to measure the binding of peptides to solubilized HLA-DR molecules. These HLA-DR molecules were chosen to allow balanced population coverage: DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*0404, DRB1*1101, DRB5*0101, DRB4*0101, DRB3*0101, DRB1*0701, DRB1*0405, DRB1*0802, DRB1*0901, DRB1*1201, and DRB1*1302 (24). MHC molecules utilized were purified from EBV transformed homozygous cell lines or single MHC allele transfected 721.221, C1R, or fibroblast lines. The cell lines were maintained by culture in RPMI-1640 medium supplemented with 2 mM L-glutaminc, 100 U (100 µg/ml) penicillin-streptomycin solution, and 10% heat-inactivated FCS. HLA-DR molecules were purified using antibody-based affinity chromatography from cell lysates prepared in 50 mM Tris-HCL, pH 8.5, containing 1% (v/v) NP-40, 150 mM NaC1, 5 mM EDTA, and 2 mM PMSF. Briefly, columns of inactivated Sepharose CL4B and Protein A Sepharose were used as pre-columns. HLA-DR molecules were captured by passage of lysates over LB3.1 monoclonal antibody (anti-HLA-DRA) columns. Antibody columns were washed with 10 mM Tris-HCL, pH8.0 with 1% (v/v) NP-40, followed by PBS containing 0.4% (w/v) n-octylglucoside. MHC molecules were then eluted with 50 mM diethylamine in 0.15 M NaC1 containing 0.4% (w/v) n-octylglucoside, pH 11.5. The pH was reduced to 8.0 and the eluates were concentrated by centrifugation in Centriprep 30 concentrators at 2000 rpm (Amicon, Beverly, MA).

[0178] HLA-DR binding assays: Radioligand binding inhibition assays were used to measure the binding of peptides to soluble HLA-DR molecules based on the inhibition of binding of a radiolabeled standard peptide as described previously (Sidney J, Southwood S, Oseroff C, del Guercio MF, Grey HM, Sette A. Measurement of MHC/pcptide interactions by gel filtration. Curr Protocols Immunol

1998;18: 18.3.2-.3.9.). Briefly, 1-10 nM of radiolabeled peptide was co-incubated for 2 days at either room temperature or 37°C with 1 μM to 1 nM purified HLA-DR molecules in the presence of a cocktail of protease inhibitors. Assays were performed at various pH conditions, ranging from pH 4 to pH 7. The final pH of assay mixtures is adjusted using citrate buffer as described elsewhere (Sidney J, Curr Protocols Immunol 1998). After incubation, the percentage of HLA-DR-bound radioactivity is determined by capturing HLA-DR/peptide complexes on Optiplates (Packard Instruments, Meriden, CT) coated with the LB3.1 antibody and determining bound counts per minute using the TopCount microscintillation counter (Packard Instruments). The amount of HLA-DR yielding 10-20% bound radioactivity is used in the inhibition assays in which the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled peptide was calculated. Under the conditions used, the measured IC50 values are reasonable approximations of the true Kd values. Competitor peptides are tested in 2-4 complete, independent experiments, at concentrations ranging from 30 μg/mL to 300 pg/mL. As in previous studies, peptides with affinities for specific HLA-DR molecules of 1000 nM or better are defined as binders for the respective antigens.

[0179] Enzyme-linked immunosorbent spot assay. A 10-day ELIspot for detecting low-frequency T cells was used to determine reactivity to the tumor antigen peptides (Table 1) as described (Knutson KL *et al.* J Clin Onc 2006;24: 4254-61). A positive response to a peptide was defined as a frequency that was significantly ($p < 0.05$, two-tailed t test) greater than the mean of control no-antigen wells and detectable (i.e., >1:100,000). PMA/Ionomycin and the CEF pool were used as positive non-tumor related controls as previously described (Knutson, 2006).

[0180] ELISA. ELISAs were done as previously described (Knutson, 2006). Briefly, 96-well plates were coated with 1 μg/ml IGFBP-2 protein, 200 ng/ml tetanus toxin or 1 μg/ml BSA. Human IgG was added at a concentration range of 200 to 0.2 ng/ml to some wells for standard curve generation. After washing and blocking, human sera were added to the plate at a 1:40 dilution in triplicate and plates were incubated for 2 hr at RT. After washing, 100 μL/well of HRP (Santa Cruz Biotechnology) was diluted 1:2000 and incubated for 1 hr at RT. After a final wash, each well was incubated with 100 μL (tetramethylbenzadine) TMB substrate (BD Bioscience). Color

development was stopped with diluted HCL and absorbance was read at 450 nm on a plate reader.

[0181] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially similar results.

Example 1

Identification of conserved HLA Class II- restricted peptides derived from tumor associated antigens using established motif search algorithms

[0182] To identify epitopes useful for vaccine design, a multidisciplinary approach was used based initially on amino acid motif searching of tumor associated antigen sequences to identify potential HLA Class II motifs (see Table I). This was followed by high throughput synthetic peptide binding assays using purified HLA molecules to determine affinity and breadth of epitope peptide binding.

[0183] Algorithm motif searches: Motif search algorithms were validated for the most common HLA Class II alleles and were focused on the HLA DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*0404, DRB1*1101, DRB5*0101, DRB4*0101, DRB3*0101, DRB1*0701, DRB1*0405, DRB1*0802, DRB1*0901, DRB1*1201, and DRB1*1302 supertypes in order to attain virtually 100% population coverage. The selected tumor associated antigen sequences were scanned for motif positive amino acid sequences using the motif definitions.

[0184] A total of about 150 Class II-restricted peptide sequences were identified that were specific for various DR supertypes (see Table I). Table I lists for each identified DR antigen peptide, the IC_{50} (nM) for each purified HLA.

Example 2

Identification of HTL epitopes for tumor vaccine inclusion

[0185] Of the peptides listed in Table I, those peptides that bound to at least 4 different HLA with an IC_{50} of less than 1000 nM were identified and are shown in Table II. The peptide sequences of Table II were further evaluated for their binding capacity to purified MHC molecules. Figures 1 through 4 show those peptides of

Table II which were immunogenic (shown in a circle). These HTL peptides are candidates for inclusion into a tumor vaccine.

[0186] Using predictive algorithms discussed above, candidate HLA-DR1-binding epitopes were identified. Binding assays targeting 15 different HLA-DR molecules revealed that 10 of the epitopes were indiscriminate, binding (IC_{50} < 1000nM) to at least four different HLA-DR variants. An interferon-gamma ELIspot assay was used to assess immunity to the indiscriminate binding peptides in 48 patients with either breast or ovarian cancer and 18 healthy controls. The results showed that elevated T cell immunity in patients was detected to several peptides (Figures 1-4).

[0187] Healthy donor and patient samples were obtained. Patients were free from active treatment for at least 30 days when blood (200 ml) was collected. For the T cell studies, the mean (± s.e.m) ages of the healthy donors and patients were 42 ± 11 and 55 ± 2 years, respectively ($p<0.0001$). Due to sera unavailability, not all of the controls used in the T cell studies were examined for tumor associated antigen antibodies in their sera. However, additional control and patient sera were available for antibody assessment. Additional healthy donor sera was obtained from Bioreclamation (Hicksville, NY).

[0188] Using an ELIspot assay with a limit of detection of approximately 1:100,000 antigen-specific T cells per million PBMC, patient-derived PBMC were screened for reactivity against all of the HLA-DR binding peptides. The immunogenicity data of several peptides is shown in Figures 1-4.

Example 3

Design and development of multi-epitope vaccines

[0189] Peptides that bound to at least 4 different HLA subtypes as described above, and were shown to be immunogenic, as shown in Figures 1-4, were selected for inclusion in a multi-epitope vaccine. Table III shows the percent of patients that demonstrated positive responses and the binding patterns to specific HLA subtypes of eight vaccine candidates.

[0190] These example and equivalents thereof will become more apparent to those skilled in the art in light of the present disclosure and the accompanying claims. It

should be understood, however, that the examples are designed for the purpose of illustration only and not limiting of the scope of the invention in any way.

(SEQ ID NOs:5-27, 20, 28-33, 19, 34-57, 57, 58-64, 64 and 65-192, respectively, in order of appearance)

TABLE I: HLA-DR binding affinity of Breast/Ovarian-derived peptides

					IC_{50} nM to purified HLA													
Peptide	Sequence	Source	Protein	Position	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1302	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0202
9019.0104	RWCIPWQRLLLTASL	CEA.10	CEA	10	1467	7860	426	193	221	107	263	4132	191	1558	2326	61	–	339	4014
9019.0105	LLTFWNPPTTAKLTI	CEA.24	CEA	24	6.9	16,313	279	52	258	3.7	174	5779	52	4995	245	46	–	2171	31
9019.0106	TAKLTIESTPFNVAE	CEA.33	CEA	33	72	613	106	41	383	70	1736	4019	5977	2907	35	140	–	33	3350
9019.0107	EVLLLVHNLPQHLFG	CEA.50	CEA	50	2.7	830	3.4	1.7	30	5.4	59	989	40	5.4	0.36	4.7	334	50	1088
9019.0108	YSWYKGERVDGNRQI	CEA.65	CEA	65	511	–	34	585	360	866	8432	–	1840	–	533	306	3002	457	1043
9019.0109	NRQGGYVIGTQQAT	CEA.76	CEA	76	216	–	108	1.5	129	46	46	345	36	4351	990	2.6	–	5.2	2230
9019.0110	GVVIGTQQATPGPAY	CEA.81	CEA	81	21		31	34	4210	1705			5677		43	836		6.3	10,223
9019.0111	GPAYSGREIIYPNAS	CEA.92	CEA	92	9435		12,989			5252					154				
9019.0112	GREIIYPNASLLIQN	CEA.97	CEA	97	62	435	251	88	550	29	1959	1355	2209	212	24	49	4035	43	10,612
9019.0113	DTGFYTLHVIKSDLV	CEA.116	CEA	116	64	984	84	260	95	23	90	83	174	174	1072	65	14,943	18	564
9019.0114	FYTLHVIKSDLVNEE	CEA.119	CEA	119	101	80	184	169	41	56	514	718	6385	616	1340	14	14,343	22	4501
9019.0005	LHVIKSDLVNEEATG	CEA.122	CEA	122	891	46	216	103	37	3893	–	–	18,399	–	394	376	–	111	–
9019.0115	KSDLVNEEATQQFRV	CEA.126	CEA	126	13,600	2580	236			6338					9963				
9019.0116	QFRVYPELPKPSIS$	CEA.137	CEA	137	1780	1727	2916			7976					786				
9019.0117	KPSISSNNSKPVEDK	CEA.146	CEA	146	405		919	2111	6959	407			–		23	97		5977	–
9019.0118	YLWWVNNQSLPVSPR	CEA.176	CEA	176	2.4	100	632	203	80	17	119	1912	22	1511	22	116	248	555	928
9019.0119	SDGVILNVLYGPDAP	CEA.226	CEA	226	113		255	314	1453	5236			9210		83	351		612	–
9019.0120	LNVLYGPDAPTISPL	CEA.231	CEA	231	331		649	1378	–	410			–		218	583		10,798	–
9019.0121	APTISPLNTSYRSGE	CEA.239	CEA	239	2431		295	49	5994	10,607			3943		125	1706		1047	51
9019.0122	QYSWFVNGTFQQSTQ	CEA.268	CEA	268	9983		21	7830	364	216			230		14,768	–		11,448	2661
9019.0123	QELFIPNITVNNSGS	CEA.282	CEA	282	147	644	25	227	379	1658	293	1086	356	1299	26	740	–	3880	3869
9019.0124	RITVTTITVYAEPPK	CEA.310	CEA	310	4115		259	697	32	649			–		2977	83		6287	14,732
9019.0125	TITVYAEPPKPFITS	CEA.315	CEA	315	12,755	539	–	12,658	5704	8230			–		2322	62		–	9255
9019.0126	YLWWVNNQSLPVSPR	CEA.354	CEA	354	1123	234	12	248	88	28	243	299	33	1121	1921	227	1088	861	2284
9019.0127	SDPVILNVLYGPDDP	CEA.404	CEA	404	384		592	347	3732	2248			5432		161	5699		9262	–
9019.0128	SYTYYRPGVNLSLSC	CEA.423	CEA	423	1.6	4425	6.8	4036	300	5.4	21	1372	776	571	7.2	46	2626	1784	135
9019.0129	YSWLIDGNIQQHTQE	CEA.447	CEA	447	1407		93			9827					1312				
9019.0130	NSGLYTCQANNSASG	CEA.471	CEA	471	49		35	37	96	8139			7338		1024	14,691		100	4717
9019.0131	RITVKTITVSAELPK	CEA.488	CEA	488	89	1267	58	54	11	4.2	3763	367	6988	2758	962	29	–	1263	1917
9019.0132	TITVSAELPKPSISS	CEA.493	CEA	493	223	74	9293	6997	3624	29	–	6871	–	–	1076	1672	–	10,349	3856
9019.0133	KPSISSNNSKPVEDK	CEA.502	CEA	502	146		403	1404	5564	121			–		4.1	138		–	–
9019.0134	YLWWVNGQSLPVSPR	CEA.532	CEA	532	1.4		2.5	1356	188	12			3195		314	182		4295	1348
9019.0135	VCGEQNSVSANRSDP	CEA.570	CEA	570	44		72	26	1854	1095			–		921	8979		1051	36
9019.0136	QNSVSANRSDPVTLD	CEA.574	CEA	574	240		511	1432	–	274			–		2.7	6657		2105	5816
9019.0137	SSYLSGANLNLSCHS	CEA.603	CEA	603	4.0		580	1596	7822	465			–		37	13,028		42	14,099
9019.0138	QYSWRINGIPQQHTQ	CEA.624	CEA	624	472		43	1203	311	11,900			151		70	2074		2286	1121
9019.0139	INGIPQQHTQVLFIA	CEA.629	CEA	629	682		181	680	942	31			–		256	8287		2700	–
9019.0140	NGTYACFVSNLATGR	CEA.650	CEA	650	839	818	11	558	30	20	70	377	2174	2052	30	2351	6963	3247	37
9019.0141	YACFVSNLATGRNNS	CEA.653	CEA	653	183	774	225	41	327	531	1774	4520	217	2399	107	1237	–	1569	17
9019.0142	NNSIVKSITVSASGT	CEA.665	CEA	665	34	103	43	1.8	128	34	184	469	209	1328	4.4	274	15,808	26	2280
9019.0143	SITVSASGTSPGLSA	CEA.671	CEA	671	2807		75	31	3374	1559			3319		592	3110		174	15,069
9019.0144	SPGLSAGATVGDMG	CEA.680	CEA	680	3507		4191	2000	2380	92			–		80	3929		2591	–
1622.03	TVGDMGVLVGVALI	CEA.688	CEA	688	384		–	–	–	4454			–		5637	7770		12,094	384
9019.0006	HQLLCCEVEIIRRAY	Cyclin D1.3	Cyclin D1	3	953	21	746	296	4200	11,553	16,299	5607	3471	5585	349	325	–	60	2010
9019.0146	DANLLNDRVLRAMLK	Cyclin D1.19	Cyclin D1	19	1463		2342			–					286				

– indicates binding affinity ≥20,000nM

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TABLE I: HLA-DR binding affinity of Breast/Ovarian-derived peptides

					IC$_{50}$ nM to purified HLA													
Peptide	Sequence	Source	Protein	Position	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1301	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB4 *0103	DRB5 *0101
9019.0147	NDRVLRAMLKAEETC	Cyclin D1.24	Cyclin D1	24	1178		1963			12,160					319				
9019.0148	RAMLKAEETCAPSVS	Cyclin D1.29	Cyclin D1	29	407	--	360	375	12,517	7793	--	5017	9145	--	16,129	15,733	--	318	1341
9019.0149	EKCVQKEVLPSMRKI	Cyclin D1.45	Cyclin D1	45	6099		3615			10,056					3587				
9019.0012	QKEVLPSMRKIVATW	Cyclin D1.49	Cyclin D1	49	9825	141	12,182	1162	3720	481	1355	1088	58	1121	700	336	--	108	2019
9019.0150	LPSMRKIVATWMLEV	Cyclin D1.53	Cyclin D1	53	8.5	826	238	28	123	4.6	1182	137	886	145	8.5	7.1	8449	50	47
9019.0151	MRKIVATWMLEVCEE	Cyclin D1.56	Cyclin D1	56	764		7953	--	1982	249			--		856	87		281	17,021
9019.0011	MLEVCEEQKCEEEVF	Cyclin D1.64	Cyclin D1	64		--													
9019.0152	EEEVFPLAMNYLDRF	Cyclin D1.74	Cyclin D1	74	850		2247	3070	2028	1597			3414		26	77		72	4444
9019.0153	VFPLAMNYLDRFLSL	Cyclin D1.77	Cyclin D1	77	146	107	2332	1567	609	8332	259	19,713	27	79	5.4	39	220	2.6	2065
9019.0007	DRFLSLEPVKKSRLQ	Cyclin D1.86	Cyclin D1	86	16	290	18	61	159	1057	37	18,378	46	4128	--	394	--	359	3.0
9019.0154	DRFLSLEPVKKSRLQ	Cyclin D1.86	Cyclin D1	86	15		60	49	253	1834			51		235	1075		227	2.6
9019.0155	LEPVKKSRLQLLGAT	Cyclin D1.91	Cyclin D1	91	102		5112	696	13,689	2152			1248		221	208		102	731
9019.0156	RLQLLGATCMFVASK	Cyclin D1.98	Cyclin D1	98	12	--	91	633	1439	468	3144	--	551	513	227	277	--	421	504
9019.0157	TCMFVASKMKETIPL	Cyclin D1.105	Cyclin D1	105	262		342	5454	1744	17			61		1631	1115		2425	36
9019.0158	ASKMKETIPLTAEKL	Cyclin D1.110	Cyclin D1	110	130		--	1992	--	220			521		530	1284		2481	2364
1622.01	TIPLTAEKLCIYTDN	Cyclin D1.116	Cyclin D1	116	253	11,708	1230	--	--	--	--	--	--	--	--	5360	--	738	253
9019.0159	KLCIYTDNSIRPEEL	Cyclin D1.123	Cyclin D1	123	1915	64	95	241	8172	1105	16,263	15,928	--	--	879	25	--	38	4620
9019.0009	DNSIRPEELLQMELL	Cyclin D1.129	Cyclin D1	129	4554	147	4672	2803	16,500	6548			--		3494	4757		87	18,258
9019.0160	DNSIRPEELLQMELL	Cyclin D1.129	Cyclin D1	129	1533		1345			3410					939				
9019.0161	PEELLQMELLLVNKL	Cyclin D1.134	Cyclin D1	134	1274		1027	348	603	401			1094		44	1119		11	2802
9019.0010	EELLQMELLLVNKLK	Cyclin D1.135	Cyclin D1	135			3386												
1622.06	LLQMELLLVNKLKWN	Cyclin D1.137	Cyclin D1	137	39	--	2539	6.4	382	2196	765	6087	157	1525	44	28	--	821	39
9019.0163	MELLLVNKLKWNLAA	Cyclin D1.140	Cyclin D1	140	46	9006	177	491	2411	1797	368	3090	48	162	1.9	246	--	39	19
9019.0164	VNKLKWNLAAAMTPHD	Cyclin D1.145	Cyclin D1	145	46	1419	88	781	747	382	702	449	1043	116	3.9	656	5867	1839	2519
9019.0165	KWNLAAAMTPHDFIEH	Cyclin D1.149	Cyclin D1	149	33	6249	3405	653	122	1301	18,625	4876	11,907	194	352	356	--	576	11
9019.0013	WNLAAAMTPHDFIEHF	Cyclin D1.150	Cyclin D1	150		6425													
9019.0166	PHDFIEHFLSKMPEA	Cyclin D1.157	Cyclin D1	157	1246		1299			1160					820				
9019.0167	NKQIRKHAQTFVALC	Cyclin D1.174	Cyclin D1	174	4.7	561	6.5	23	25	4.4	195	286	34	5.9	2.9	3.3	342	8.7	23
9019.0168	AQTFVALCATDVKFI	Cyclin D1.182	Cyclin D1	182	8.4	551	26	133	55	15	445	536	276	132	219	97	--	46	18
9019.0169	VKFISNPPSMVAAGS	Cyclin D1.193	Cyclin D1	193	6.7	4128	12	28	117	304	319	752	88	21	24	84	5280	290	826
9019.0170	FPSMVAAGSVVAAVQ	Cyclin D1.199	Cyclin D1	199	6.8	--	12	26	1718	133	1401	301	466	10,090	55	26	--	93	957
9019.0171	VAAVQGLNLRSPNNF	Cyclin D1.209	Cyclin D1	209	44	18,558	226	532	4248	161	10,850	--	11,089	2918	171	1205	--	296	3541
9019.0172	IEALLESSLRQAQQN	Cyclin D1.251	Cyclin D1	251	2698		18			--					12,776				
9019.0014	EALLESSLRQAQQNM	Cyclin D1.252	Cyclin D1	252	8212	151	605	1019	12,905	--			756		4755	8176		4028	--
9019.0024	LAALCRWGLLLALLP	Her2/neu.3	Her2/neu	3	2044		5894			7005					704				
9019.0025	WGLLLALLPPGAAST	Her2/neu.9	Her2/neu	9	1687		750	0.93	219	16,366			12		521	21		33	337
1622.02	LLALLPPGAASTQVC	Her2/neu.12	Her2/neu	12	17		5146	118	--	7283			944		10,697	17,445		10,806	17
9019.0027	GTDMKLRLPASPETH	Her2/neu.28	Her2/neu	28	2117		1698			6811					14,523				
9019.0028	RHLYQGCQVVQGNLE	Her2/neu.47	Her2/neu	47	1435		422			4948					5043				
9019.0029	GCQVVQGNLELTYLP	Her2/neu.52	Her2/neu	52	2600		2739			1972					417				
9019.0030	NLELTYLPTNASLSF	Her2/neu.59	Her2/neu	59	4.9	7356	6.2	2.7	38	7.2	94	3055	30	141	105	23	--	29	189
9019.0031	LTYLPTNASLSFLQD	Her2/neu.62	Her2/neu	62	9.7	3364	19	36	80	15	426	4083	213	156	47	132	141	1622	173
9019.0032	IQEVQGYVLIAHNQV	Her2/neu.77	Her2/neu	77	57	7763	111	178	102	55	213	302	165	3438	103	75	13,508	546	1361
9019.0033	YVLIAHNQVRQVPLQ	Her2/neu.83	Her2/neu	83	28	454	53	104	1185	92	300	358	208	302	1.9	679	649	124	18
9019.0034	HNQVRQVPLQRLRIV	Her2/neu.88	Her2/neu	88	950	971	840	78	1303	80	85	6644	21	42	270	340	--	18	173

-- indicates binding affinity >20,000nM.

TABLE I: HLA-DR binding affinity of Breast/Ovarian-derived peptides

Peptide	Sequence	Source	Protein	Position	IC_{50} nM to purified HLA														
					DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1352	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101
9019.0035	VRQVPLQRLRIVRGT	Her2/neu.91	Her2/neu	91	3065		1798			218					2546				
9019.0001	GTQLFEDNYALAVLD	Her2/neu.104	Her2/neu	104	210	20	640	3923	14,921	129			9195		4.0	450		6955	3744
9019.0036	GDPLNNTTPVTGASP	Her2/neu.120	Her2/neu	120	6356		7468			15,172					1030				
9019.0037	TTPVTCASPGGLREL	Her2/neu.126	Her2/neu	126	992		2447	695	13,198	1843			11,554		605	17,148		2319	3001
9019.0038	PGGLRELQLRSLTEI	Her2/neu.134	Her2/neu	134	732		3397	564	110	1541			2207		786	4405		2245	16,275
9019.0039	TEILKGGVLIQRNPQ	Her2/neu.146	Her2/neu	146	51		40	12	769	2486			692		343	1185		513	1878
9019.0040	GGVLIQRNPQLCYQD	Her2/neu.151	Her2/neu	151	142		198	93	3845	14,279			1720		34	806		351	13,612
9019.0041	NPQLCYQDTILWKDI	Her2/neu.158	Her2/neu	158	3653		368			--					5074				
9019.0042	ICELHCPALVTYNTD	Her2/neu.263	Her2/neu	263	101		754	136	1627	1324			521		3216	748		441	2809
9019.0043	STDVGSCTLVCPLHN	Her2/neu.305	Her2/neu	305	2872		--			2139					6781				
9019.0044	CYGLGMEHLREVRAV	Her2/neu.342	Her2/neu	342	139	265	1027	493	5122	271	--	--	3064	18,979	25	690	--	444	3776
9019.0045	MEHLREVRAVTSANI	Her2/neu.347	Her2/neu	347	9.6	2970	533	12	200	9.7	95	4345	262	221	23	86	--	81	216
9019.0046	LREVRAVTSANIQEF	Her2/neu.350	Her2/neu	350	17	3913	43	8.2	50	12	456	5187	661	161	1.5	27	--	165	94
9019.0047	CKKIFGSLAFLPESF	Her2/neu.367	Her2/neu	367	119		121	171	316	45			3080		206	655		316	4567
9019.0048	PESFDGDPASNTAPL	Her2/neu.378	Her2/neu	378	10,101	989	381	9030	1724	2825			12,591		3905	805		654	--
9019.0049	TAPLQPEQLQVFETL	Her2/neu.389	Her2/neu	389	1112	3674	2633			6994					2322				
9019.0050	ITGYLYISAWPDSLP	Her2/neu.406	Her2/neu	406	96		243	1771	8.5	136			2573		751	152		270	67
9019.0051	PDSLPDLSVFQNLQV	Her2/neu.416	Her2/neu	416	--		--			--					--				
9019.0052	LSVFQNLQVIRGRIL	Her2/neu.422	Her2/neu	422	1.3	345	6.5	33	26	7.1	148	859	9.6	466	80	33	--	67	17
9019.0053	NLQVIRGRILHNGAY	Her2/neu.427	Her2/neu	427	1.9	6879	971	206	3394	12	119	4217	173	47	9.9	3.3	4320	486	28
9019.0054	RGRILHNGAYSLTLQ	Her2/neu.432	Her2/neu	432	2.4	710	489	129	2845	5.6	5077	430	773	40	1.3	5.4	358	562	82
9019.0055	SLTLQGLGISWLGLR	Her2/neu.442	Her2/neu	442	93		145	116	409	3096			5307		1597	50		22	--
9019.0056	GLGISWLGLRSLREL	Her2/neu.447	Her2/neu	447	50	122	41	163	3.1	55	1538	1140	2268	436	3030	5.2	--	154	2048
9019.0057	LRSLRELGSGLALIH	Her2/neu.455	Her2/neu	455	7.1	--	896	14	603	142	1075	554	309	498	16	24	16,142	549	726
9019.0058	GLALIHHNTHLCFVH	Her2/neu.464	Her2/neu	464	465		434	171	177	277			2822		18	164		357	6081
9019.0059	NTHLCFVHTVPWDQL	Her2/neu.471	Her2/neu	471	416		796	207	116	178			793		175	1481		117	7390
9019.0060	DECVGEGLACHQLCA	Her2/neu.502	Her2/neu	502	459		1968	3405	2267	2097			4976		123	6267		1117	12,273
9019.0061	GHCWGPGPTQCVNCS	Her2/neu.518	Her2/neu	518	1913		3740			6742					2912				
9019.0062	SQFLRGQECVEECRV	Her2/neu.532	Her2/neu	532	462		1020	4595	--	540			16,562		146	285		1525	7043
9019.0063	FCRVLQGLPREYVNA	Her2/neu.543	Her2/neu	543	262		786	9551	812	4861			254		549	685		2572	201
9019.0064	LQGLPREYVNARHCL	Her2/neu.547	Her2/neu	547	354	8995	201	1144	663	1903			405		670	423		2551	49
9019.0065	PSGVKPDLSYMPIWK	Her2/neu.601	Her2/neu	601	1832	1578	1525			2688					1531				
9019.0066	ASPLTSISAVVGIL	Her2/neu.648	Her2/neu	648	15	10,905	29	36	712	24	3089	118	511	1431	14	59	18,926	1589	206
9019.0067	ISAVVGILLVVVLGV	Her2/neu.655	Her2/neu	655	5153		420	2996	447	941			5158		443	6005		3109	--
9019.0068	ILLVVVLGVVFGILI	Her2/neu.661	Her2/neu	661	--		635	3532	376	780			2464		478	7117		11,015	--
9019.0069	VLGVVFGILIKRRQQ	Her2/neu.666	Her2/neu	666	67	2449	177	335	101	17	35	--	12	268	17	185	--	958	38
9019.0070	QQKIRKYTMRRLLQE	Her2/neu.679	Her2/neu	679	303		3782	5306	--	44			40		14	2.0		93	300
9019.0071	IRKYTMRRLLQETEL	Her2/neu.682	Her2/neu	682	665		2706	2305	1050	722			46		2583	1.6		303	2207
9019.0072	MRILKETELRKVKVL	Her2/neu.712	Her2/neu	712	266		3193	1266	8030	176			2103		1318	329		368	1223
9019.0073	ETELRKVKVLGSGAF	Her2/neu.717	Her2/neu	717	284	19,518	246	27	645	101	139	5423	176	2472	959	205	--	452	1020
9019.0074	KVKVLGSGAFGTVYK	Her2/neu.722	Her2/neu	722	64		810	204	10,552	77			1711		1308	244		1380	1369
9019.0075	GENVKIPVAIKVLRE	Her2/neu.743	Her2/neu	743	491		1065	488	2093	180			4535		1149	729		84	8353
1622.03	KIPVAIKVLRENTSP	Her2/neu.747	Her2/neu	747	1505		3856	125	1178	--			4106		501	611		7805	1505
9019.0077	AYVMAGVGSPYVSRL	Her2/neu.771	Her2/neu	771	92		164	171	596	45			1738		402	24		5060	308
9019.0078	MAGVGSPYVSRLLGI	Her2/neu.774	Her2/neu	774	2050		1651			352					1508				

-- indicates binding affinity >20,000nM.

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Peptide	Sequence	Source	Protein	Position	IC$_{50}$ nM to purified HLA													
					DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1303	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101
9019.0079	SRLLGICLTSTVQLV	Her2/neu:783	Her2/neu	783	89	2923	85	13	90	90	634	137	86	446	4.7	59	3567	451	392
9019.0080	TVQLVTQLMPYGCLL	Her2/neu:793	Her2/neu	793	164		215	433	4826	1288			11,126		3594	9.4		16	8002
9019.0081	RGRLGSQDLLNWCMQ	Her2/neu:814	Her2/neu	814	1039		1412			2029					3367				
9019.0082	LLNWCMQIAKGMSYL	Her2/neu:822	Her2/neu	822	944		558	195	1094	380			159		2082	799		8359	92
9019.0083	CMQIAKGMSYLEDVR	Her2/neu:826	Her2/neu	826	959		2651	867	1040	116			405		2232	1902		3005	130
9019.0002	GMSYLEDVRLVHRDL	Her2/neu:832	Her2/neu	832	123	27	957	1357	4815	5408			244		3789	567		2543	467
9019.0084	VRLVHRDLAARNVLV	Her2/neu:839	Her2/neu	839	59	1503	105	248	561	356			16		57	1097		298	3425
9019.0085	HRDLAARNVLVKSPN	Her2/neu:843	Her2/neu	843	158		401	765	11,210	1332			1648		116	221		525	302
9019.0086	ARNVLVKSPNHVKIT	Her2/neu:848	Her2/neu	848	65	1275	209	197	10,536	118	717	4771	513	628	15	29	16,142	742	4.9
9019.0087	PIKWMALESILRRRF	Her2/neu:885	Her2/neu	885	12	30	14	259	161	664	312	3620	133	66	349	3.3	--	62	3.4
9019.0003	IKWMALESILRRRFT	Her2/neu:886	Her2/neu	886	16	10	37	1975	435	1795	515	9282	136	241	1118	11	--	340	3.3
9019.0088	ESILRRRFTHQSDVW	Her2/neu:892	Her2/neu	892	165		794	7977	2575	9598			1159		16,279	2726		818	5805
9019.0089	SDVWSYGVTVWELMT	Her2/neu:903	Her2/neu	903	165		2760	3621	1900	546			2639		467	1879		--	5423
9019.0090	GVTVWELMTFGAKPY	Her2/neu:909	Her2/neu	909	40		377	4.1	2107	558			315		8868	20		1171	34
9019.0091	VWELMTFGAKPYDGI	Her2/neu:912	Her2/neu	912	36		676	144	4704	191			1952		7553	245		3565	65
9019.0092	AKPYDGIPAREIPDL	Her2/neu:920	Her2/neu	920	116		--	--	12,548	41			--		19,575	6957		--	3944
1622.04	ICTIDVYMIMVKCWM	Her2/neu:946	Her2/neu	946	--		717	--	955	--			1489		--	2652		--	--
9019.0094	DVYMIMVKCWMIDSE	Her2/neu:950	Her2/neu	950	1312		189	7807	274	297			179		395	1303		697	716
9019.0095	RPRFRELVSEFSRMA	Her2/neu:966	Her2/neu	966	25	6218	38	62	151	369	376	2321	125	1779	12,182	348	--	351	26
9019.0096	FRELVSEFSRMARDP	Her2/neu:969	Her2/neu	969	20	150	22	51	1487	5085	1714	--	331	--	--	124	4537	1262	380
9019.0004	FSRMARDPQRFVVIQ	Her2/neu:976	Her2/neu	976	29	35	512	2224	855	1423	798	1481	49	6867	240	1498	901	227	45
9019.0097	DGDLGMGAAKGLQSL	Her2/neu:1087	Her2/neu	1087	110		254	566	5799	828			2233		3234	1684		896	2141
9019.0098	AKGLQSLPTHDPSPL	Her2/neu:1095	Her2/neu	1095	149		194	19	3864	4459			6806		3991	1665		572	--
9019.0099	LQRYSEDPTVPLPSE	Her2/neu:1109	Her2/neu	1109	1367	38	25	13,089	226	367			94		280	5310		--	--
9019.0100	PEYVNQPDVRPQPPS	Her2/neu:1137	Her2/neu	1137	6165		150			--					1714				
9019.0101	EGPLPAARPAGATLE	Her2/neu:1154	Her2/neu	1154	17,288		--			7463					10,247				
9019.0102	GATLERPKTLSPGKN	Her2/neu:1164	Her2/neu	1164	1812		2792			--					5332				
9019.0103	KDVFAFGGAVENPEY	Her2/neu:1182	Her2/neu	1182	1595		--			1577					16,146				
9019.0173	LPRVGCPALPLPPPP	IGFBP2.2	IGFBP2	2	1906		6543			--					8856				
9019.0174	ALPLPPPPLLPLLPL	IGFBP2.9	IGFBP2	9	174	--	18	4.2	20	336	1087	--	41	1337	1524	262	--	40	9665
9019.0175	PPPLLPLLPLLLLLL	IGFBP2.14	IGFBP2	14	15	5816	15	16	65	13	86	507	121	25	1322	5.0	16,239	2.1	121
9019.0176	LLPLLPLLLLLLGAS	IGFBP2.17	IGFBP2	17	119	--	337	35	674	964	213	5893	458	320	2622	182	--	19	2390
9019.0177	PLLLLLLGASGGGGG	IGFBP2.22	IGFBP2	22	20	18,088	339	2144	1422	5852	5696	--	12,818	18,255	2164	253	--	316	--
9019.0178	AEVLFRCPPCTPERL	IGFBP2.39	IGFBP2	39	1285		1611			35,979					5774				
9019.0179	PERLAACGPPPVAPP	IGFBP2.50	IGFBP2	50	28		5545	165	4853	--			3541		3818	4054		4455	19,685
9019.0180	PPPVAPPAAVAAVAG	IGFBP2.58	IGFBP2	58	178	--	380	81	--	2084	--	317	--	--	237	1578	--	1886	--
9019.0181	GARMPCAELVREPGC	IGFBP2.73	IGFBP2	73	164		11,638	13,072	--	--			2965		16,279	--		4697	--
9019.0015	CAELVREPGCGCCSV	IGFBP2.78	IGFBP2	78		12,298													
9019.0016	CARLEGEACGVYTPR	IGFBP2.93	IGFBP2	93		--													
9019.0182	ELPLQALVMGEGTCE	IGFBP2.121	IGFBP2	121	1502		6782			15,638					9167				
9019.0017	QALVMGEGTCEKRED	IGFBP2.125	IGFBP2	125		2240													
9019.0183	SEGGLVFNHVDSTMN	IGFBP2.157	IGFBP2	157	1153		1196			4623					2518				
9019.0184	TMNMLGGGGSAGRKP	IGFBP2.168	IGFBP2	169	1021		791			6177					5686				
9019.0185	LKNGMKFLAVFREKV	IGFBP2.184	IGFBP2	184	607	--	883	562	34	--	260	--	163	1765	4974	91	--	417	843
9019.0186	KFLAVFREKVTEQHR	IGFBP2.189	IGFBP2	189	2045		28			--					--				

-- indicates binding affinity ≥20,000nM.

TABLE I: HLA-DR binding affinity of Breast/Ovarian-derived peptides

					IC_{50} nM to purified HLA													
Peptide	Sequence	Source	Protein	Position	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1302	DRB1 *1501	DRB3 *0202	DRB4 *0101	DRB5 *0101
9019.0018	ELAVFREKVIEQHRQ	IGFBP2.190	IGFBP2	190		8621													
9019.0019	REKVIEQHRQMGKGG	IGFBP2.195	IGFBP2	195		2839													
9019.0187	GKHHLGLEEPKKLRP	IGFBP2.209	IGFBP2	209	238		1654	2756	5494	3697			16.749		3629	3345		1378	6390
9019.0020	KHHLGLEEPKKLRPP	IGFBP2.210	IGFBP2	210		4016													
9019.0188	EPKKLRPPARIPCQ	IGFBP2.217	IGFBP2	217	1258		806			1122					1215				
9019.0189	LDQVLERISTMRLPD	IGFBP2.234	IGFBP2	234	795	452	25	20	18	5.2	1607	52	467	314	1052	84	–	367	223
9019.0021	TMRLPDERGPLEHLY	IGFBP2.248	IGFBP2	248		--													
9019.0190	ERGPLEHLYSLHIPN	IGFBP2.249	IGFBP2	249	7.7	--	497	29	110	18	9361	638	1993	59	1149	23	--	1648	4090
9019.0191	GLYNLKQCKMSLNGQ	IGFBP2.268	IGFBP2	268	923	--	743	2835	5589	1791	204	9434	2600	413	561	223	--	1616	5609
9019.0192	TGKLIQGAPTIRGDP	IGFBP2.293	IGFBP2	293	148	9554	40	27	608	883	5169	700	7989	2691	927	1733	2191	465	36
9019.0022	APTIRGDPECHLFYN	IGFBP2.300	IGFBP2	300	4051	31	1643	2908	--	16.779	--	--	--	16,917	1674	3669	191	2510	--
9019.0023	CHLFYNEQQEARGVH	IGFBP2.309	IGFBP2	309	2490	162	1600	3187	--	--	--	--	--	1855	146	--	18.902	5310	--

-- indicates binding affinity ≥20,000nM

(SEQ ID NOs:6-10,13-15,20,25,20,29,32,40-42, 46,51-52, 56-57, 59,67-70,73-77,86-90,102-103,109,111,114,126,136,145-146,156,165-168, 171,179,186 and 188-190, respectively, in order of appearance)

TABLE II: Breast/Ovarian HLA-DR Supertypo Candidates

					IC_{50} nM to purified HLA													
Peptide No.	Sequence	Source	Protein	Position	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1302	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101	Total XRN
9019.0105	LLTFWNPPTTAKLTI	CEA.24	CEA	24	6.9	16,313	273	52	258	3.7	176	5779	52	8995	245	66	--	2171	31	10
9019.0106	TAKLTIESTPFNVAE	CEA.33	CEA	33	72	613	106	41	383	79	1736	4019	5977	2907	35	140	--	53	3350	9
9019.0107	SVLLLVHNLPQHLFG	CEA.50	CEA	50	2.7	830	3.4	1.7	30	5.4	59	989	40	5.4	0.36	4.7	334	50	1088	14
9019.0108	YSWYKGERVDGNRQI	CEA.65	CEA	65	511	--	34	585	360	866	8432	--	1840	--	533	306	3002	453	1043	8
9019.0109	NRQDGYVIGTQQAT	CEA.76	CEA	76	216	--	108	1.5	129	46	46	245	36	4251	690	2.6	--	5.2	2226	11
9019.0112	GREITYPNASLLIQN	CEA.97	CEA	97	62	433	251	88	550	29	1950	1353	2209	212	24	49	4035	43	10,812	10
9019.0113	DTGFVTLHVIKSDLV	CEA.116	CEA	116	66	984	85	260	95	23	90	83	174	174	1077	65	14,943	18	564	13
9019.0114	FYTLHVIKSDLVNEE	CEA.119	CEA	119	101	80	184	169	41	56	514	718	6385	616	1340	14	14,343	22	4501	11
9019.0118	YLWWVNNQSLPVSPR	CEA.176	CEA	176	2.4	100	832	203	80	17	119	1912	22	1511	32	116	248	559	928	13
9019.0123	QELFIPNITVNNSGS	CEA.282	CEA	282	147	644	25	227	379	1658	293	1086	356	1299	26	740	--	3580	3869	9
9019.0126	YLWWVNNQSLPVSPR	CEA.354	CEA	354	1123	234	12	248	88	28	243	299	33	1121	1921	227	1088	861	2284	10
9019.0128	SYTYYRPGVNLSLSC	CEA.423	CEA	423	1.6	4425	6.8	4036	300	5.4	21	1372	776	371	7.2	46	2626	1784	135	10
9019.0131	RTTVKTITVTAELPK	CEA.488	CEA	488	89		58	54	11	4.2			6984.0		962	29		1261	1917	7
9019.0140	NGTYACFVSNLATGR	CEA.650	CEA	650	839	818	11	558	30	20	70	377	2174	2032	30	2351	6965	3247	37	10
9019.0141	YACFVSNLATGRNNS	CEA.653	CEA	653	183	774	225	41	327	531	1774	4520	217	2399	107	1237	--	1569	17	9
9019.0142	NNSIVKSITVSASGT	CEA.665	CEA	665	34	103	43	1.8	128	34	184	469	209	1328	4.4	274	15,808	26	2280	12
9019.0006	HQLLCCSVETRRAY	Cyclin D1.3	Cyclin D1	3	953	23	716	256	4200	11,553	16,297	5607	3471	5585	549	325	--	60	2016	7
9019.0012	QKEVLPSMRKIVATW	Cyclin D1.49	Cyclin D1	49	9625	111	12,182	1101	3720	481	1155	1088	58	1121	700	938	--	108	2015	6
9019.0150	LPSMRKIVATWMLEV	Cyclin D1.53	Cyclin D1	53	8.5	826	238	28	123	4.6	1182	137	886	345	8.5	7.1	8,449	50	47	13
9019.0153	VFPLAANYLDRFLSL	Cyclin D1.77	Cyclin D1	77	146	107	2132	1567	609	3332	258	19,713	27	79	5.4	39	239	2.6	2005	10
9019.0007	DRFLSLEPVKKSRLQ	Cyclin D1.86	Cyclin D1	86	16	290	18	61	159	1057	37	18,378	46	4128	--	391	--	359	3.0	10
9019.0156	RLQLLQATCMFVASK	Cyclin D1.98	Cyclin D1	98	12	--	91	633	1439	468	3144	--	831	513	227	277	--	421	304	10
1622.06	LLQMELLLVNKLKWN	Cyclin D1.137	Cyclin D1	137	39	--	3539	6.4	332	2196	765	6097	187	1325	44	28	--	321	39	9
9019.0163	MELLLVNKLKWNLAA	Cyclin D1.140	Cyclin D1	140	46	9006	177	491	2411	1797	368	3090	46	162	4.9	246	--	39	19	10
9019.0164	VNKLKWNLAAMTPHD	Cyclin D1.145	Cyclin D1	145	46	1419	88	781	747	382	702	440	1043	116	3.0	356	5887	1939	2919	10
9019.0165	KWNLAAMTPHDFIER	Cyclin D1.149	Cyclin D1	149	33	6249	3405	653	122	1101	18,625	4876	11,907	194	352	356	--	576	41	8
9019.0167	NEQDRKHAQTFVAL	Cyclin D1.174	Cyclin D1	174	4.7	561	6.5	23	25	4.4	195	236	34	3.9	2.0	3.3	342	8.7	23	15
9019.0168	AQTFVALCATDVKFI	Cyclin D1.182	Cyclin D1	182	8.4	551	26	133	59	13	445	536	276	332	219	97	--	46	18	14
9019.0169	VKFIDKFPFSMVAAGS	Cyclin D1.193	Cyclin D1	193	6.7	4128	12	18	117	304	319	752	88	21	24	84	5299	290	626	13
9019.0170	PFSMVAAGSVVAAVQ	Cyclin D1.199	Cyclin D1	199	6.8	--	12	26	1718	133	1401	301	466	10,090	59	26	--	91	959	10
9019.0171	VAAVQGLNLRSPNNF	Cyclin D1.209	Cyclin D1	209	44	18,558	226	532	4248	161	10,850	--	11,085	2018	171	1205	--	296	3541	6
9019.0030	NLELTYLPTNASLSF	Her2/neu.59	Her2/neu	59	4.9	7356	6.2	2.7	38	7.2	94	3055	30	141	105	23	--	29	189	12
9019.0031	LTYLPTNASLSFLQD	Her2/neu.62	Her2/neu	62	9.7	3364	19	16	80	15	436	1081	213	350	47	132	141	1633	173	12
9019.0032	IQEVQGYVLIAHNQV	Her2/neu.77	Her2/neu	77	57	7763	111	178	102	35	213	302	165	3438	103	75	13,508	348	1361	11
9019.0033	YVLIAHNQVRQVPLQ	Her2/neu.83	Her2/neu	83	28	434	53	104	1185	92	300	358	208	302	1.9	679	649	124	18	14
9019.0034	HNQVRQVPLQRLRIV	Her2/neu.88	Her2/neu	88	950	971	840	78	1303	80	85	6644	21	42	270	340	--	18	178	12
9019.0035	MSELRLVRAVTSANI	Her2/neu.347	Her2/neu	347	9.6	2970	533	12	200	9.7	95	4345	262	221	25	86	--	81	216	12
9019.0046	LREVRAVTSANIQEF	Her2/neu.350	Her2/neu	350	17	3913	43	8.2	50	12	436	5187	661	161	1.5	27	--	163	94	12
9019.0052	LSVTQNLQVERGRIL	Her2/neu.422	Her2/neu	422	1.3	345	6.3	33	26	7.1	148	859	9.6	486	80	35	--	67	17	14
9019.0054	RGRILHNGAYSLTLQ	Her2/neu.432	Her2/neu	432	2.4	710	480	129	2845	5.6	5077	430	773	40	1.3	5.4	358	562	82	13
9019.0057	LRSLRELGSGLALIH	Her2/neu.455	Her2/neu	455	7.1	--	896	14	603	142	1075	594	309	498	16	24	16,142	548	726	12
9019.0069	VLGVVFGILIKRRQQ	Her2/neu.666	Her2/neu	666	67	2449	177	335	101	17	35	--	12	268	17	185	--	938	38	12
9019.0079	SRLLGICLTSTVQLV	Her2/neu.783	Her2/neu	783	80	2923	83	13	90	9.0	634	137	80	446	4.7	39	3567	481	392	13
9019.0087	PICWMALESILRRRF	Her2/neu.885	Her2/neu	885	12	30	14	250	161	664	312	3620	133	66	349	3.3	--	62	3.4	13
9019.0003	ICWAALESILRRRFT	Her2/neu.886	Her2/neu	886	16	10	32	1075	435	1795	515	9282	136	241	1118	11	--	340	3.3	10

-- indicates binding affinity ≥30,000nM.

RECTIFIED SHEET (RULE 91)

WO 2009/059011
PCT/US2008/081799
CA 02704397 2010-04-30

TABLE II: Breast/Ovarian HLA-DR Supertype Candidates

Peptide No.	Sequence	Source	Protein	Position	IC_{50} nM to purified HLA														Total XRN
					DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1301	DRB1 *1302	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101	
9019.0001	FSRMARDPQRFVVIQ	Her2/neu976	Her2/neu	976	29	35	512	2214	855	1423	798	1481	49	6867	240	1408	901	227	45	10
9019.0174	ALPLPFPPLLPLLPL	IGFBP2.9	IGFBP2	9	174	--	18	42	20	336	1087	--	41	1337	1524	262	--	40	9665	8
9019.0175	PPPLLPLLPLLLLLL	IGFBP2.14	IGFBP2	14	15	5816	15	16	95	13	86	307	121	23	1822	5.0	16,239	2.1	121	12
9019.0176	LLPLLPLLLLLLGAS	IGFBP2.17	IGFBP2	17	119	--	367	55	674	964	214	5895	458	320	2022	182	--	19	2389	10
9019.0177	PLLLLLLGASGGGGG	IGFBP2.22	IGFBP2	22	20	18,833	369	2144	1422	5882	5696	--	12,818	18,255	2164	253	--	316	--	4
9019.0180	PPPVAPPAAVAAVAG	IGFBP2.58	IGFBP2	58	178	--	380	81	--	2684	--	317	--	--	287	1378	--	1888	--	5
9019.0185	LKSGSRELAVERKKV	IGFBP2.184	IGFBP2	184	607	--	881	862	34	--	269	--	165	1768	4974	91	--	417	843	9
9019.0189	LDQVLERISTMRLPD	IGFBP2.234	IGFBP2	234	795	452	25	20	18	5.2	1607	52	467	514	1052	84	--	367	223	12
9019.0190	ERGPLEHLYSLHIPN	IGFBP2.249	IGFBP2	249	77	--	497	29	110	18	8361	658	1983	50	1149	23	--	1048	4000	8
9019.0191	GLYNLKQCKMSLNGQ	IGFBP2.268	IGFBP2	268	923	--	743	2515	5539	1791	204	9434	2809	413	561	225	--	1613	5809	6
9019.0192	TGKLIQGAPTIRGDP	IGFBP2.293	IGFBP2	293	148	9554	40	27	608	883	5160	700	7989	2691	927	1733	2191	465	36	9

-- indicates binding affinity $\geq$20,000nM.

TABLE III Vaccine Candidates

EPITOPE	%	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1302	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101
HER2/NEU.59	15	X		X	X	X	X	X		X	X	X	X		X	X
HER2/NEU.885	25	X	X	X	X	X		X		X	X	X	X		X	X
CEA.24	17	X		X	X	X	X	X		X		X	X			X
CEA.653	25	X		X	X	X				X		X				X
IGFBP2.17	19	X		X	X			X		X	X		X		X	
IGFBP2.249	23	X		X	X	X	X				X		X			
CYCLIND1.53	13	X		X	X	X	X		X		X	X	X		X	X
CYCLIND1.199	13	X		X	X		X		X	X		X	X		X	

%=Percent of patients with responses

What is Claimed Is:

1. A composition comprising the isolated HLA-DR binding peptide of SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109.

2. The composition of claim 1, comprising the isolated HLA-DR binding peptide of SEQ ID NO:145 and two or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109.

3. The composition of claim 1, comprising the isolated HLA-DR binding peptide of SEQ ID NO:145 and three or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109.

4. The composition of claim 1, comprising SEQ ID NO:145, SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109.

5. The composition of any one of claims 1 to 4, further comprising a CTL peptide.

6. The composition of any one of claims 1 to 5, further comprising a lipid.

7. The composition of claim 6, wherein the lipid is attached to the HLA-DR binding peptide.

8. The composition of claim 1, wherein one or more of said peptides is linked to a spacer molecule.

9. The composition of any one of claims 1 to 8, further comprising a carrier.

10. The composition of any one of claims 1 to 8, further comprising an antigen presenting cell.

11. An isolated nucleic acid encoding the isolated HLA-DR binding peptide of SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the

group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109.

12. The isolated nucleic acid of claim 11, further encoding at least one additional peptide or signal sequence.

13. The isolated nucleic acid of claim 12, wherein said at least one additional peptide or signal sequence is a further HLA-DR binding peptide.

14. The isolated nucleic acid of claim 12, wherein said at least one additional peptide or signal sequence is a CTL peptide.

15. An isolated nucleic acid encoding the isolated HLA-DR binding peptide of SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, and at least one protein or polypeptide included to enhance or decrease immunogenicity, wherein the at least one protein or polypeptide is a cytokine, a cytokine-inducing molecule, or a costimulatory molecule.

16. A composition comprising the nucleic acid of any one of claims 11 to 15 and a pharmaceutical excipient.

17. The composition of any one of claims 1 to 10 for use in preventing or treating cancer in a patient.

18. The nucleic acid of any one of claims 11 to 15 for use in preventing or treating cancer in a patient.

19. The peptide of SEQ ID NO:145 in combination with one or more peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, for use as a diagnostic reagent to determine treatment, prognosis, or risk of cancer in a patient.

20. An isolated polynucleotide that is a multi-epitope construct comprising between five and fifty-five nucleic acids each encoding an HTL epitope, wherein the HTL

epitopes comprise SEQ ID NO:145 and one or more epitopes selected from the group consisting of SEQ ID NOS:86, 89, 90, and 109, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame.

21. The isolated polynucleotide of claim 20, further comprising one or more HTL epitopes selected from the group consisting of CEA p24, CEA p75, CEA p176, CEA p423, CEA p488, CEA p650, CEA p663, IGFBP-2 p17, IGFBP-2 p22, IGFBP-2 p249, IGFBP-2 p293, Cyclin D1 p3, Cyclin D1 p49, Cyclin D1 p53, Cyclin D1 p98, Cyclin D1 p137, Cyclin D1 p145, Cyclin D1 p174, and Cyclin D1 p199.

22. An isolated polynucleotide selected from the group consisting of:

(a) a multi-epitope construct comprising between five and fifty-five nucleic acids each encoding an HTL epitope, wherein the HTL epitope is any one of the epitopes listed in Table I and comprises SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame;

(b) a multi-epitope construct comprising between five and fifty-five nucleic acids each encoding an HTL epitope, wherein the HTL epitope is any one of the epitopes listed in Table II and comprises SEQ ID NO:145 and one or more additional HLA-DR binding peptides selected from the group consisting of SEQ ID NO:86, SEQ ID NO:89, SEQ ID NO:90, and SEQ ID NO:109, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame;

(c) a multi-epitope construct comprising five and eight nucleic acids each encoding an HTL epitope, wherein the HTL epitope is any one of the epitopes listed in Table III and comprises SEQ ID NO:145 and SEQ ID NO:86, and wherein the nucleic acids are directly or indirectly joined to one another in the same reading frame;

(d) the multi-epitope construct of (a) to (c), wherein one or more spacer nucleic acids are positioned between the HTL epitope nucleic acids of (a) or (b) or (c);

(e) the multi-epitope construct of (d), wherein said one or more spacer nucleic acids each encode 1 to 8 amino acids;

(f) the multi-epitope construct of (d), wherein one or more of said spacer nucleic acid encodes an amino acid sequence selected from the group consisting of: an amino acid sequence comprising or consisting of GPGPG (SEQ ID NO:193), an amino acid sequence comprising or consisting of PGPGP (SEQ ID NO:194), an amino acid sequence comprising or consisting of (GP)n, an amino acid sequence comprising or consisting of (PG)n, an amino acid sequence comprising or consisting of (GP)nG, and an amino acid sequence comprising or consisting of (PG)nP, where n is an integer between zero and eleven;

(g) the multi-epitope construct of any one of (a) to (f), further comprising one or more MHC Class II targeting nucleic acids;

(h) the multi-epitope construct of (g), wherein said one or more targeting nucleic acids encode one or more targeting sequences selected from the group consisting of : an Ig kappa signal sequence, a tissue plasminogen activator signal sequence, an insulin signal sequence, an endoplasmic reticulum signal sequence, a LAMP-1 lysosomal targeting sequence, a LAMP-2 lysosomal targeting sequence, an HLA-DM lysosomal targeting sequence, an HLA-DM-association sequence of HLA-DO, an Ig-a cytoplasmic domain, an Ii protein, an influenza matrix protein, an HCV antigen, and a yeast Ty protein;

(i) the multi-epitope construct of any one of (a) to (h), wherein the construct is optimized for HTL epitope processing;

(j) the multi-epitope construct of any one of (a) to (i), wherein said HTL nucleic acids are sorted to minimize the number of HTL junctional epitopes encoded therein;

(k) the multi-epitope construct of any one of (a) to (j), wherein said multiepitope construct comprises at least two Carcinoembryonic Antigen (CEA) HTL epitopes, at least two Cyclin D1 HTL epitopes, at least two Human Epidermal Growth Factor Receptor 2 (HER-2/neu) HTL epitopes, and at least two Insulin Growth Factor Binding Protein 2 (IGFBP-2) HTL epitopes;

(l) the multi-epitope construct of (k), wherein at least two Carcinoembryonic Antigen (CEA) HTL epitopes, at least two Cyclin D1 HTL epitopes, at least two Human Epidermal Growth Factor Receptor 2 (HER-2/neu) HTL epitopes, and at least two Insulin

Growth Factor Binding Protein 2 (IGFBP-2) HTL epitopes are selected from the epitopes listed in Table II; and

(m) the multi-epitope construct of any one of (a) to (l), wherein said multi-epitope construct consists of the epitopes listed in Table III.

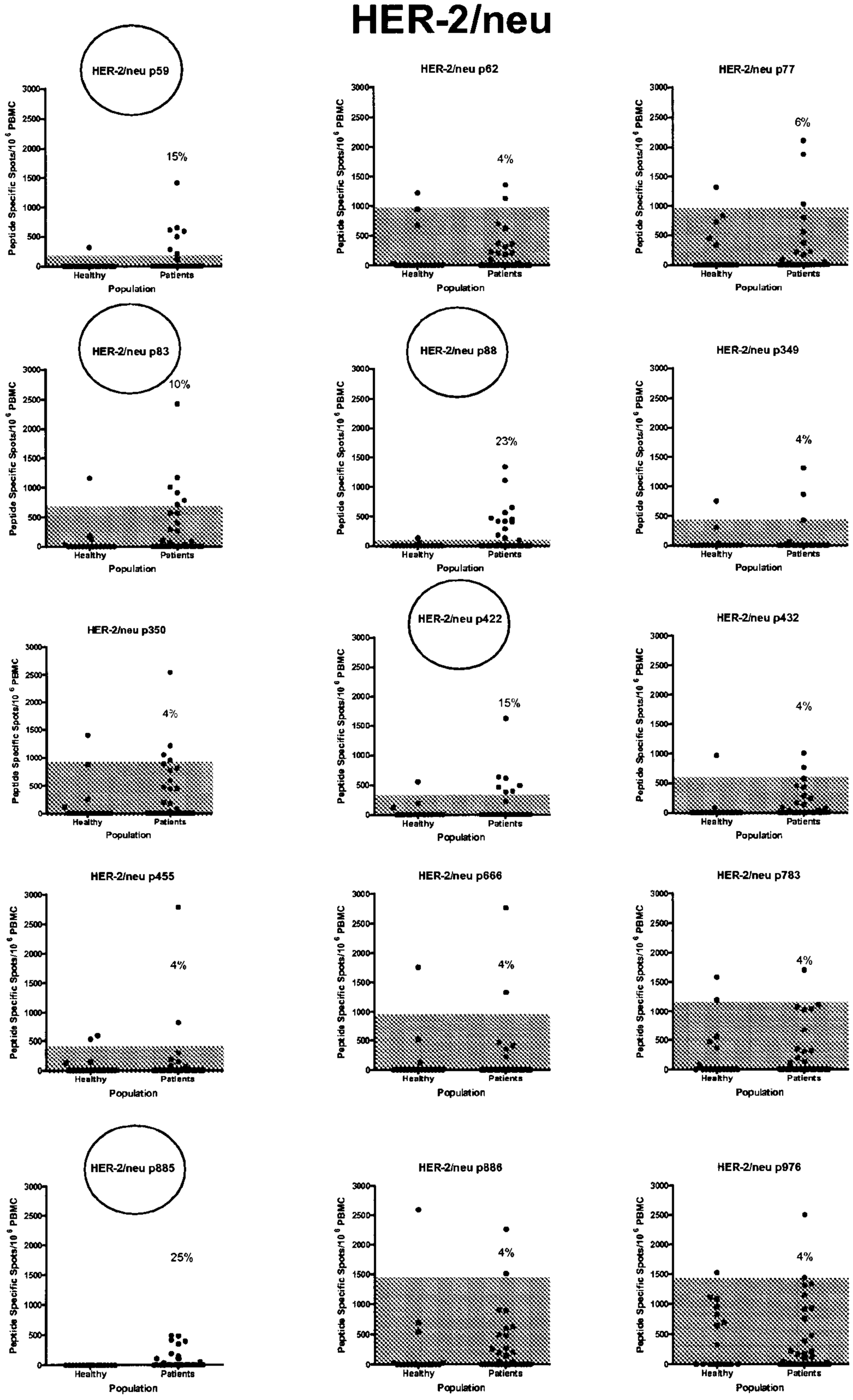
HER-2/neu
HER-2/neu p59
15%
HER-2/neu p62
4%
HER-2/neu p77
6%
HER-2/neu p83
10%
HER-2/neu p88
23%
HER-2/neu p349
4%
HER-2/neu p350
4%
HER-2/neu p422
15%
HER-2/neu p432
4%
HER-2/neu p455
4%
HER-2/neu p666
4%
HER-2/neu p783
4%
HER-2/neu p885
25%
HER-2/neu p886
4%
HER-2/neu p976
4%
Peptide Specific Spots/10 6 PBMC
Healthy
Patients
Population

Figure 1

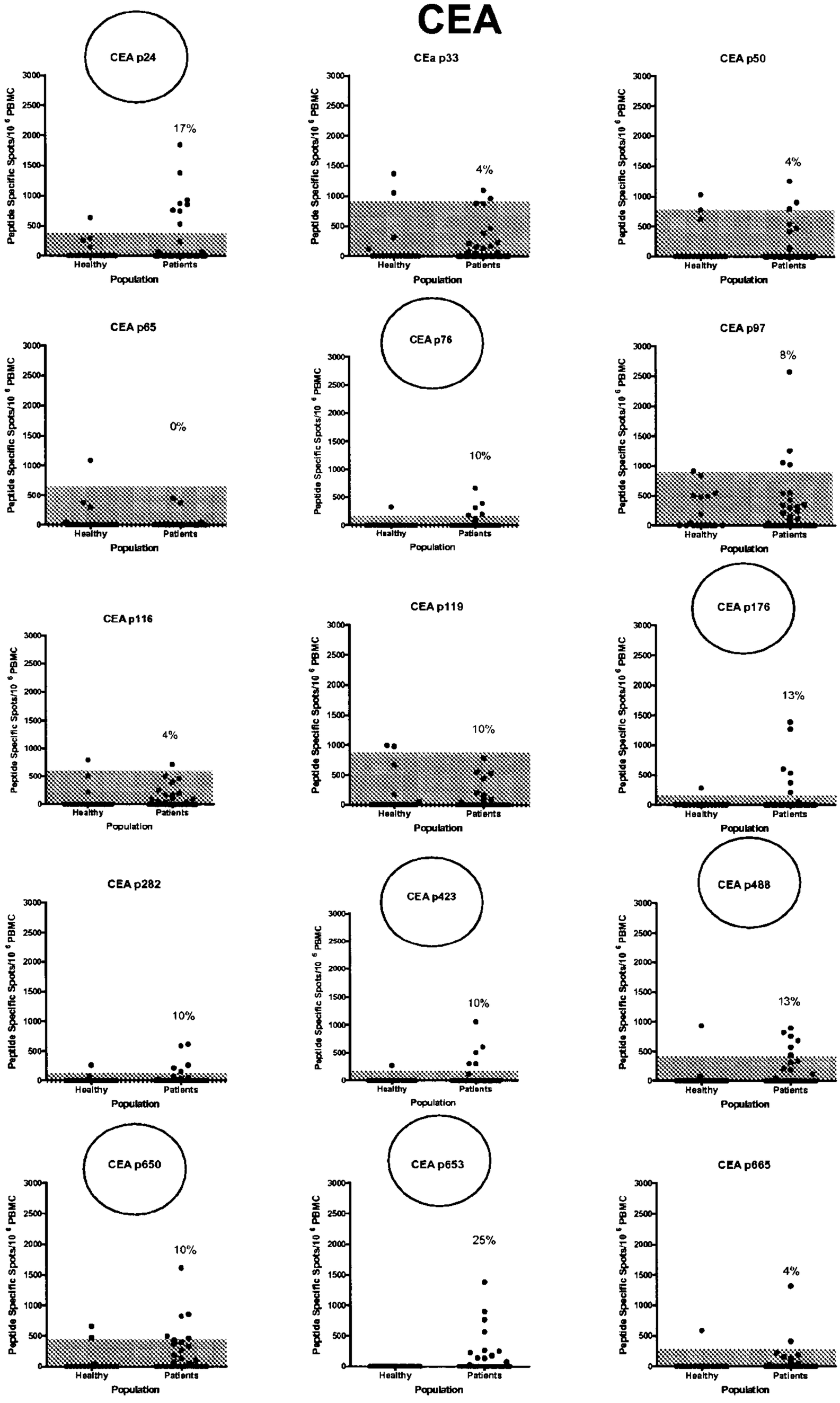
CEA
CEA p24
17%
CEa p33
4%
CEA p50
4%
CEA p65
0%
CEA p76
10%
CEA p97
8%
CEA p116
4%
CEA p119
10%
CEA p176
13%
CEA p282
10%
CEA p423
10%
CEA p488
13%
CEA p650
10%
CEA p653
25%
CEA p665
4%
Peptide Specific Spots/10 6 PBMC
Healthy
Patients
Population

Figure 2

IGFBP-2

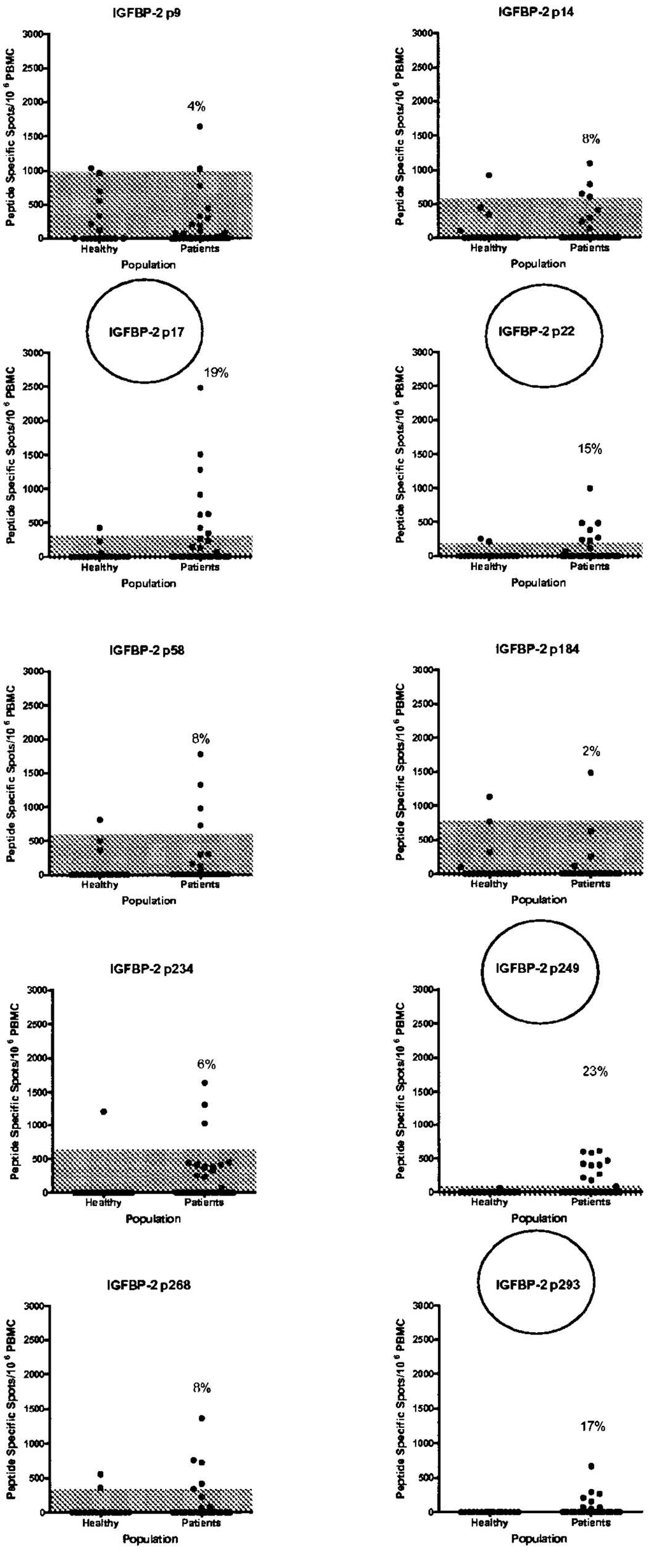

Figure 3

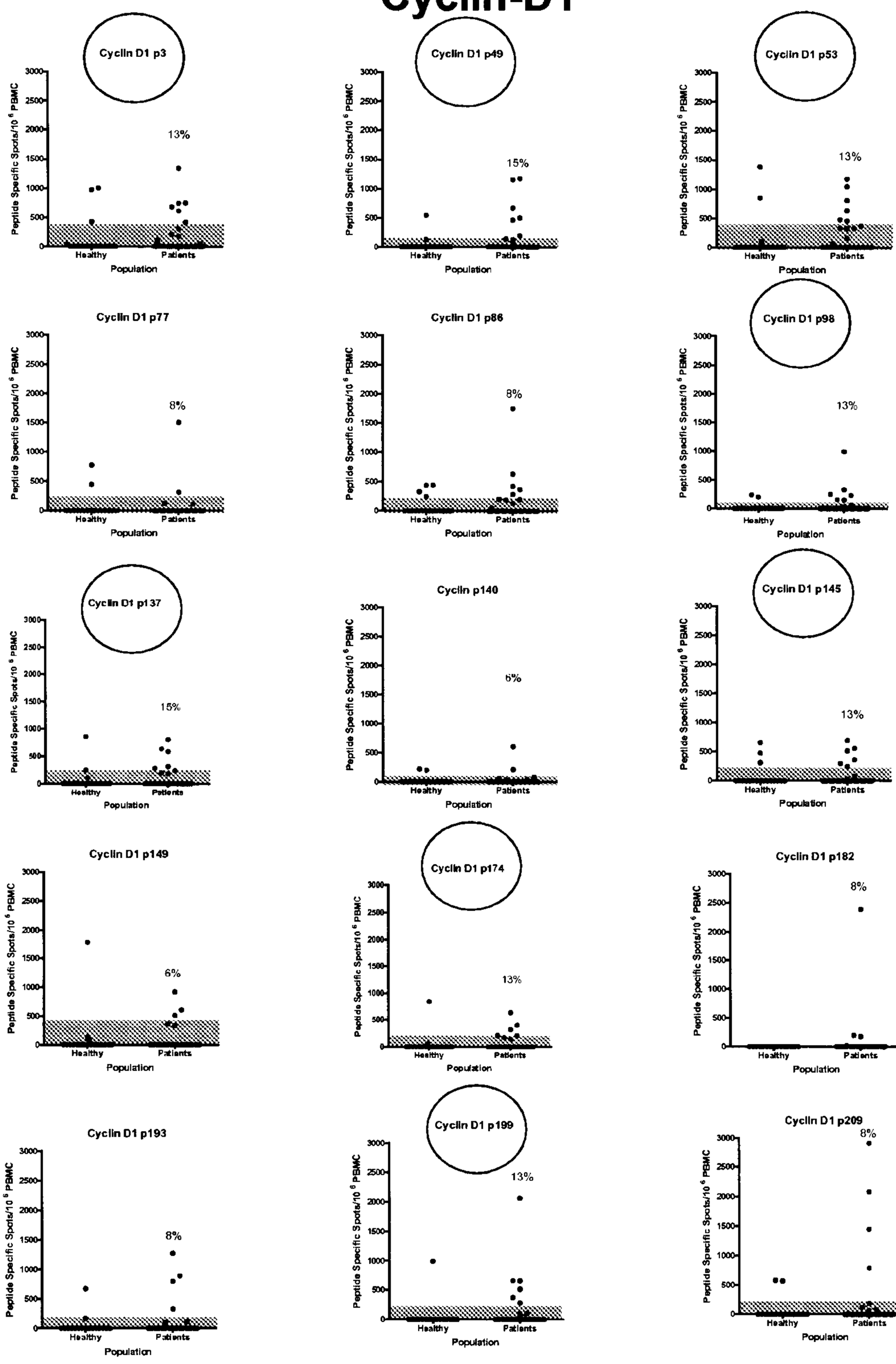
Cyclin-D1
Cyclin D1 p3
13%
Cyclin D1 p49
15%
Cyclin D1 p53
13%
Cyclin D1 p77
8%
Cyclin D1 p86
8%
Cyclin D1 p98
13%
Cyclin D1 p137
15%
Cyclin p140
6%
Cyclin D1 p145
13%
Cyclin D1 p149
6%
Cyclin D1 p174
13%
Cyclin D1 p182
8%
Cyclin D1 p193
8%
Cyclin D1 p199
13%
Cyclin D1 p209
8%
Peptide Specific Spots/10 6 PBMC
Healthy
Patients
Population
99% confidence interval

Figure 4

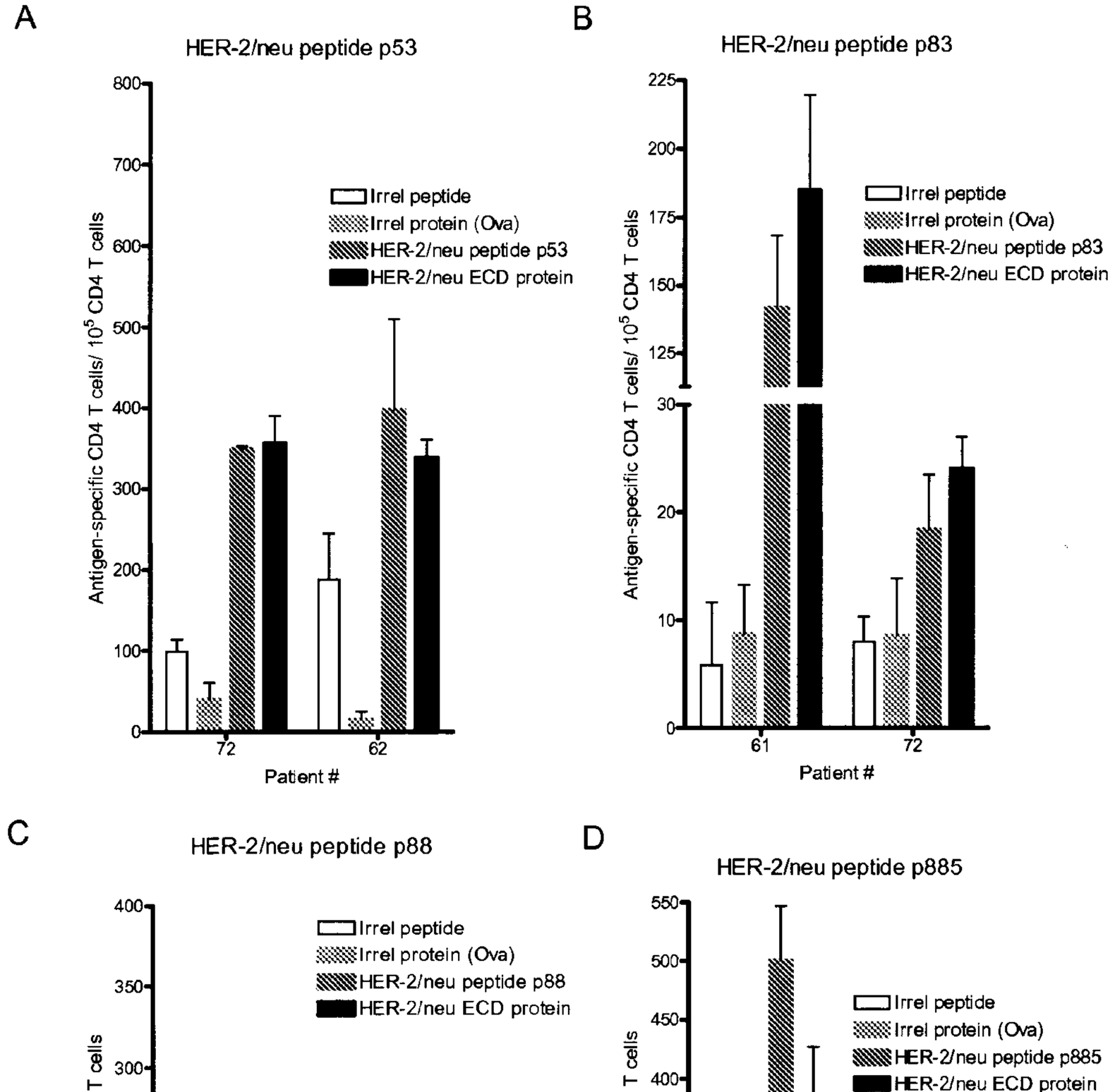
A
HER-2/neu peptide p53
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Irrel peptide
Irrel protein (Ova)
HER-2/neu peptide p53
HER-2/neu ECD protein
Patient #
72
62
B
HER-2/neu peptide p83
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Irrel peptide
Irrel protein (Ova)
HER-2/neu peptide p83
HER-2/neu ECD protein
Patient #
61
72
C
HER-2/neu peptide p88
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Irrel peptide
Irrel protein (Ova)
HER-2/neu peptide p88
HER-2/neu ECD protein
Patient #
61
62
D
HER-2/neu peptide p885
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Irrel peptide
Irrel protein (Ova)
HER-2/neu peptide p885
HER-2/neu ECD protein
Patient #
77
61

Figure 5

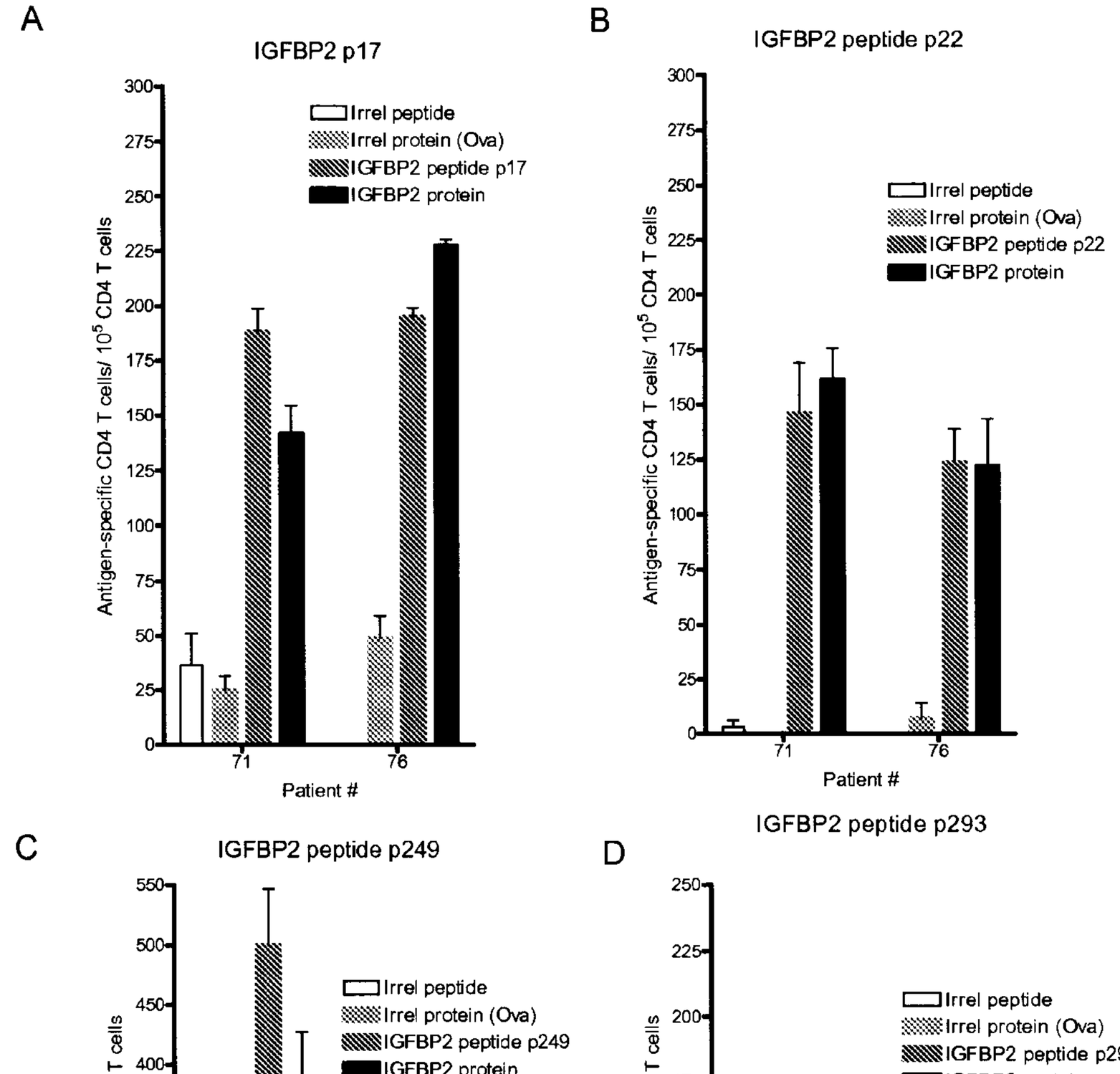
A
IGFBP2 p17
Irrel peptide
Irrel protein (Ova)
IGFBP2 peptide p17
IGFBP2 protein
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Patient #
B
IGFBP2 peptide p22
Irrel peptide
Irrel protein (Ova)
IGFBP2 peptide p22
IGFBP2 protein
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Patient #
C
IGFBP2 peptide p249
Irrel peptide
Irrel protein (Ova)
IGFBP2 peptide p249
IGFBP2 protein
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Patient #
D
IGFBP2 peptide p293
Irrel peptide
Irrel protein (Ova)
IGFBP2 peptide p293
IGFBP2 protein
Antigen-specific CD4 T cells/ 10^5 CD4 T cells
Patient #

Figure 6