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(54) Title: PAN-DR BINDING POLYPEPTIDES AND USES THEREOF

(57) **Abstract**: The present invention provides novel artificial oligopeptides capable of binding HLA Class II molecules encoded by several alleles. The oligopeptides include the sequence $AX_1FVAAX_2TLX_3AX_4A$ (SEQ ID NO : 1), wherein X_1 is selected from the group consisting of W, F, Y, H, D, E, N, Q, I and K; X_2 is selected from the group consisting of F, N, Y and W; X_3 is selected from the group consisting of H and K, and X_4 is selected from the group consisting of A, D and E, with the proviso that the oligopeptide sequence is not AKFV AAWTLKAAA. The invention also relates to larger peptides comprising the oligopeptides, polynucleotides encoding the oligopeptides and larger peptides, as well as to compositions comprising the oligopeptides, peptides or polynucleotides. Also disclosed are methods for inducing immune responses.

PAN-DR BINDING POLYPEPTIDES AND USES THEREOF

BACKGROUND OF THE INVENTION

Immunogenic peptides, containing epitopes recognized by T helper cells, have been found to be useful in inducing immune responses. The use of helper peptides to enhance antibody
5   responses against particular determinants is described for instance in Hervas-Stubbs, *et al.,* *Vaccine* 12:867-871 (1994).

Although allele-specific polymorphic residues that line the peptide binding pockets of MHC alleles tend to endow each allele with the capacity to bind a unique set of peptides, there are instances in which a given peptide has been shown to bind to more than one MHC allele. For
10   example, several investigators reported degenerate binding and/or recognition of certain epitopes in the context of multiple DR types, leading to the concept that certain peptides might represent "universal" epitopes (Busch, *et al., Int. Immunol.* **2**:443-451 (1990); Panina-Bordignon, *et al., Eur. J. Immunol.* **19**:2237-2242 (1989); Sinigaglia, *et al., Nature* **336**:778-780 (1988); O'Sullivan, *et al., J. Immunol.* **147**:2663-2669 (1991); Roache, *et al.,*
15   *J. Immunol.* **144**:1849-1856 (1991); Hill, *et al., J. Immunol.* **147**:189-197 (1991)). Pan-DR binding peptides have been described in, for example, US Patent No. 6,413,935; WO 95/07707; WO/2005/120563; and Alexander, *et al., Immunity* **1**:751-761 (1994). These peptides have been shown to help in the generation of various immune responses against antigens.

20   BRIEF SUMMARY OF THE INVENTION

The present invention is based on studies of the effects of selected mutations in the sequence of PADRE (SEQ ID NO: 3) in terms of providing immunogenic peptides (both in the form of short peptides, oligopeptides and polypeptides) having improved stability vs. proteolytic enzymes.

25   The invention thus relates to an isolated polypeptide comprising or consisting of an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, such as at least 50 nM , wherein the oligopeptide sequence comprises $AX_1FVAAX_2TLX_3AX_4A$ (SEQ ID NO:1), wherein

X_1 is selected from the group consisting of W, F, Y, H, D, E, N, Q, I and K;

X_2 is selected from the group consisting of F, N, Y and W;

X_3 is selected from the group consisting of H and K, and

X_4 is selected from the group consisting of A, D and E,

5   with the proviso that the oligopeptide sequence is not AKFVAAWTLKAAA (SEQ ID NO: 3).

The invention further relates to polynucleotides encoding the polypeptides of the invention and to vectors including such polynucleotides. Also encompassed by the invention are cells comprising the polynucleotides of the invention. The invention further relates to compositions, including pharmaceutical compositions, comprising the polypeptides or
10   oligopeptides or polynucleotides or vectors or cells of the invention. Finally, the invention relates to a method of stimulating an immune response by administering a polypeptide or oligopeptide or polynucleotide or vector or cell or composition of the invention to a subject in need thereof.

BRIEF DESCRIPTION OF THE DRAWING

15   Fig. 1 shows data from binding experiments determining the HLA-DR binding of peptides of the invention to various HLA-DR molecules, cf. Example 1 for details.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this
20   invention belongs. This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

The terms "oligopeptide" or "peptide" as used herein refer to a chain of at least four amino acid residues or amino acid mimetics. The oligopeptides or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in salt forms, and either free of modifications, including but not limited to, glycosylation, side chain oxidation, or

5   phosphorylation or containing one or more of these modifications. While optional modifications do not destroy the biological activity of the polypeptides described herein, however, the invention includes options in which the modifications reduce or eliminate biological activity (e.g., to limit activity until such modification if removed, e.g., *in vivo*).

The terms "polypeptide," and "protein" are used interchangeably herein to refer to a polymer

10   of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by

15   covalent peptide bonds. The terms further include polypeptides which have undergone post-translational modifications, for example, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids.

Also included as polypeptides of the present invention are fragments, derivatives, analogs, or

20   variants of the foregoing polypeptides, and any combination thereof. Polypeptides, and fragments, derivatives, analogs, or variants thereof can be antigenic and immunogenic polypeptides.

The terms "amino acid residue," "amino acid" and "residue" when referring to an amino acid residue in a peptide, oligopeptide or protein are used interchangeably and, as used herein,

25   mean an amino acid or amino acid mimetic joined covalently to at least one other amino acid or amino acid mimetic through an amide bond or amide bond mimetic.

As used herein, the term "amino acid," when unqualified, refers to an "L-amino acid" or L-amino acid mimetic.

As used herein, the nomenclature used to describe peptide compounds follows the

30   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. When amino acid residue positions are referred to in an epitope, they are numbered in an amino to carboxyl direction with position one being the position closest to the amino terminal end of

the epitope, or the peptide or protein of which it may be a part. In the amino acid structure formulae, 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.

As used herein, the symbols for the amino acids are shown below.

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

The term "pan DR-binding peptide" or "pan-DR binding epitope" as used herein refers to a member of a family of molecules that binds more than one MHC class II DR molecule (e.g., binding each of the more than one MHC molecule with an IC_{50} of less than 100 nM, such as at

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least 50 nM). In some embodiments, the pan DR-binding oligopeptides of the present invention are peptides capable of binding at least 2, 3, 4, 5, 6, 7, 8, 9, 10,, 11, or all 12 most common DR alleles (DR1, 2w2b, 2w2a, 3, 4w4, 4w14, 5, 7, 52a, 52b, 52c, and 53). The pan-DR binding oligopeptides of the present invention, in addition to promoting an immune response against a second determinant, can also serve as target immunogens themselves. Thus, for instance, in the case in which the pan-DR binding peptide itself is linked to a carbohydrate epitope, the immune response may be to both the pan-DR binding peptide and the carbohydrate epitope.

As used herein, the term "PADRE" refers to the pan DR binding peptide having the amino acid sequence AKFVAAWTLKAAA (SEQ ID NO: 3).

As used herein, the expression "PADRE analogue" refers to a pan DR peptide comprising an amino acid sequence which, compared to PADRE, includes at most 1, 2, 3 or 4 amino acid changes, of which at least one is made in K2, W7, K10 or A12.

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. Assays for determining binding are described in detail, e.g., in PCT publications WO 94/20127 and WO 94/03205, the disclosure of each which is herein incorporated by reference. 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.,* 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, for instance PADRE. Although as a particular assay becomes more, or less, sensitive, the IC_{50} value of the peptides tested may change somewhat, the binding relative to the reference peptide will not significantly 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. 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, 19990; 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

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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), and measurement of class I MHC
5   stabilization or assembly (*e.g.*, Ljunggren *et al.*, *Nature 346*:476, 1990; Schumacher *et al.*, *Cell 62*:563, 1990; Townsend *et al.*, *Cell 62*:285, 1990; Parker *et al.*, *J. Immunol. 149*:1896, 1992).

The term "expression" refers to the biological production of a product encoded by a coding sequence. In most cases a DNA sequence, including the coding sequence, is transcribed to
10   form a messenger-RNA (mRNA). The messenger-RNA is then translated to form a polypeptide product which has a relevant biological activity. Also, the process of expression may involve further processing steps to the RNA product of transcription, such as splicing to remove introns, and/or post-translational processing of a polypeptide product.

The term "construct" as used herein generally denotes a composition that does not occur in
15   nature. A construct can be produced by synthetic technologies, e.g., recombinant DNA preparation and expression or chemical synthetic techniques for nucleic or amino acids. A construct can also be produced by the addition or affiliation of one material with another such that the result is not found in nature in that form. A "multi-epitope construct" can be used interchangeably with the term "minigene" or "multi-epitope nucleic acid vaccine," and
20   comprises multiple epitope nucleic acids that encode peptide epitopes of any length that can bind to a molecule functioning in the immune system, e.g., in some embodiments, a MHC class I and a T-cell receptor and/or a MHC class II and a T-cell receptor. Epitope nucleic acids in a multi-epitope construct can encode, for example, class II MHC epitopes or a combination of class I MHC epitopes and class II MHC epitopes.

25   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 vitro* or *in vivo*, an epitope is the collective features of a molecule, such as primary, secondary and tertiary
30   peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor or MHC molecule. Thus, the term "epitope" includes, but is not limited to, immunogenic peptides of the invention capable of binding to an appropriate MHC molecule and thereafter inducing a cytotoxic T cell response, or a helper T cell response, or alternatively, capable of binding an antibody, and thereafter inducing an antibody response to
35   the antigen from which the immunogenic peptide is derived.

A "flanking residue" is a residue that is positioned next to an epitope. A flanking residue can be introduced or inserted at a position adjacent to the N-terminus or the C-terminus of an epitope.

The terms "immunogen" and "antigen" are used interchangeably and mean any compound to which a cellular or humoral immune response is to be directed against. Furthermore, antigenic or immunogenic peptides of the invention may be linear, i.e., be comprised of contiguous amino acids in a polypeptide, or, in the case of antibody-epitopes, may be three dimensional or conformational, i.e., where a functional epitope is comprised of non-contiguous amino acids which come together due to the secondary or tertiary structure of the polypeptide, thereby forming an epitope.

As used herein, the term "antigenic determinant" is any structure that can elicit, facilitate, or be induced to produce an immune response, for example carbohydrate epitopes, lipids, proteins, peptides, or combinations thereof.

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or genes, that is capable of interacting with and binding to a specified protein or antigen contained in a composition comprising, but not limited to, one or more proteins and/or antigens.

The term "CTL epitope" refers to a peptide, which is recognized and bound by a particular MHC class I molecule, and which is recognized by a T lymphocyte when complexed with the particular MHC Class I molecule. In some embodiments, the CTL epitope can be from about 8 to about 13 amino acids in length, from about 9 to about 11 amino acids in length, or from about 9 to about 10 amino acids in length.

The term "HTL epitope" refers to a peptide, which is recognized and bound by a particular MHC class II molecule, and which is recognized by a T lymphocyte when complexed with the particular MHC Class II molecule. In some embodiments, the HTL epitope can be from about 6 to about 30 amino acids in length, from about 8 to about 30 amino acids in length, from about 10 to about 30 amino acids, from about 12 to about 30 amino acids in length, from about 6 to about 25 amino acids in length, from about 8 to about 25 amino acids in length, from about 10 to about 25 amino acids, from about 12 to about 25 amino acids in length, from about 6 to about 18 amino acids in length, from about 8 to about 18 amino acids in length, from about 10 to about 18 amino acids, or from about 12 to about 18 amino acids in length.

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When stating that a T lymphocyte "recognizes" a complex between an MHC molecule and a CTL or HTL epitope is herein mean that the T-cell receptors on the T lymphocyte bind to the complex with the effect that the T lymphocyte is activated.

The terms "identical" or percent "identity," in the context of two or more peptide sequences,
5   refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using a sequence comparison algorithm or by manual alignment and visual inspection.

An example of an algorithm that is suitable for determining percent sequence identity and
10   sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of
15   length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative
20   alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum
25   achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino
30   acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The phrases "isolated" or "biologically pure" refer to material which is substantially or
35   essentially free from components which normally accompany it as found in its native state.

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The term "linker" as used herein is any compound used to provide covalent linkage and spacing between two functional groups (e.g., a pan-DR binding peptide and a desired immunogen). In some embodiments, the linker comprises neutral molecules, such as aliphatic carbon chains, amino acids or amino acid mimetics, which are substantially

5   uncharged under physiological conditions and may have linear or branched side chains. In some cases, the linker can, itself, be immunogenic. Various linkers useful in the invention are described in more detail, below. Additionally, the verbs "link" and "conjugate" are used interchangeably herein and refer to covalent attachment of two or more species.

The terms "link" or "join" refers to any method known in the art for functionally connecting

10   peptides, including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, and electrostatic bonding.

The term "directly linked" or "directly joined" refers to being joined without anything intervening. For example, in the case of two peptides being directly joined, one peptide would be joined or bonded to another peptide, as described above, without any sequence, molecule,

15   spacer, linker, etc. intervening between the two peptides. Directly joined peptides may or may not share amino acids in common, i.e., the sequences of the two peptides are allowed to not overlap and also to overlap; the latter is e.g. relevant if a PADRE analogue of the invention is introduced in another (poly)peptide by means of insertion – in order to ensure the presence of the PADRE analogue's amino acid sequence, it is in only necessary to insert

20   those amino acid residues which together with the amino acid residues at the insertion point provides for the entire sequence for the PADRE analogue.

The term "indirectly linked" refers to being joined with something intervening. For example, in the case of two peptides being indirectly joined, one peptide would be joined or bonded to another peptide, as described above, with a sequence, molecule, spacer, linker, etc.

25   intervening between the two peptides.

"Major Histocompatibility Complex" or "MHC" is a cluster of genes that 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, e.g., Paul, Fundamental Immunology, 3rd ed., Raven Press, New York,

30   1993.

The phrase "pharmaceutically acceptable" refers to a generally non-toxic, inert, and/or physiologically compatible composition.

DETAILED DESCRIPTION

I.   Introduction

The inventors have discovered a new class of peptides that are pan-DR binders, i.e., the
peptides bind to MHC class II molecules encoded by a number of different DR alleles, thereby
5   allowing the peptides to stimulate an immune response in a wide spectrum of individuals.
Further, the inventors have discovered that a number of the new peptides have significantly
improved protease resistance, thereby allowing for a longer *in vivo* half-life. Surprisingly,
some of these peptides reflect a greatly increased ability to boost an immune response over
known pan-DR peptides.

10   A biological activity of the polypeptides of the invention (or fragments thereof) is the ability
to bind an appropriate MHC molecule and induce a T helper response, which optionally helps
to induce an immune response against a target immunogen or immunogen mimetic. In the
case of peptides useful for stimulating antibody responses, the peptides of the invention will
induce a T helper response, which in turn helps induce a humoral response against the target
15   immunogen.

II.   Pan-DR binding peptides

The present invention provides a new class of peptides that are pan-DR binding peptides and
provides uses thereof. The peptides of the present invention are capable of binding more than
one of a number of different DR alleles and thus find use in increasing immune responses to
20   various antigens/immunogens. The peptides of the present invention further find use in
eliciting an enhanced immune response over known pan-DR binding peptides, for example, as
disclosed in US 5,736,142.

As demonstrated herein, the inventors have identified a series of peptide sequences that bind
to MHC molecules encoded by multiple HLA alleles. Partly in view of this data, the inventors
25   have determined that the sequence motifs below represent pan-DR binding peptides with
improved activity and immunogenicity. In some embodiments, the polypeptides of the
present invention comprise an oligopeptide sequence that can bind an MHC molecule encoded
by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more different DR alleles, wherein the
oligopeptide sequence comprises $AX_1FVAAX_2TLX_3AX_4A$ (SEQ ID NO:1), wherein X_1 is selected
30   from the group consisting of W, F, Y, H, D, E, N, Q, I and K; X_2 is selected from the group
consisting of F, N, Y and W; X_3 is selected from the group consisting of H and K, and X_4 is
selected from the group consisting of A, D and E. In some embodiments, the polypeptide

comprises an oligopeptide sequence that can bind an MHC molecule encoded by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more different DR alleles, wherein the oligopeptide sequence comprises $AX_1FVAAX_2TLHAAA$ (SEQ ID NO:2), wherein X_1 is selected from the group consisting of Y, H, I, E, N, Q and K; and X_2 is selected from the group consisting of F, N, Y and W. According to the inventions, the oligopeptide sequence does not comprise AKFVAAWTLKAAA (SEQ ID NO:3).

In some embodiments, the polypeptides of the present invention comprise one or more copies of one or more amino acid sequences constituting the 13 amino acid C-terminal fragment of an oligopeptide selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, or SEQ ID NO: 72.

The pan-DR binding peptides of the present invention are capable of binding multiple different MHC alleles and can also be referred to as "helper peptides." In some embodiments, the pan-DR binding peptides are capable of binding MHC molecules encoded by 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more different MHC class II alleles. In some embodiments the response induced is an enhanced immune response. In some embodiments immune response is directed towards the pan-DR binding oligopeptides of the invention. Alternatively, or in addition, the binding of the pan-DR binding oligopeptides to an MHC molecule further enhances an immune response to a second antigen, e.g., a polypeptide comprising one or more CTL peptides and/or a humoral (i.e., antibody) response to a protein or non-protein antigen.

The pan-DR polypeptide sequences described herein can be used alone, or as part of a larger fusion protein or conjugate. Thus, the polypeptides of the invention can include additional amino acids at either or both termini in addition to the pan-DR peptide sequences set forth

herein. For example, it can be desirable to include other amino acid sequences as tags or markers or to promote *in vivo* or *in vitro* stability or otherwise to include additional benefits as is generally understood in the art. Moreover, the polypeptides of the invention can comprise one, two, three or more copies of one or more pan-DR oligopeptide sequence in the

5   polypeptide and/or include multiple different pan-DR oligopeptide sequences in the polypeptides of the invention. Moreover, the polypeptides can include at least one additional HTL oligopeptide and/or CTL oligopeptide and/or antibody-inducing polypeptide, for example where it is desirable to enhance the immune response to such sequences or to polypeptides comprising such sequences. In such embodiments, the pan-DR sequences described herein

10 function as alternatives to traditional carrier proteins such as Keyhole Limpet hemocyanin, tetanus toxoid, and diphtheria toxoid or as alternatives for prior art universal T-helper epitopes.

The polypeptides of the invention can be of any length. In some embodiments, the polypeptides comprising at least one pan-DR binding oligopeptide sequence of the invention,

15 and optionally other amino acid sequences, are no longer than e.g., 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80, 60, 50, 40, 30, 20, or 15 amino acids, but in many cases the polypeptides comprising the pan-DR binding oligopeptide(s) are of approximately the same length as a native polypeptide, which has been modified by introduction of one or a few of the pan-DR binding oligopeptides of the inventionn (so that the polypeptide includes a

20 majority of a native polypeptide sequence). Some polypeptides of the invention including pan-DR binding oligopeptides of the invention are, on the other hand, "multi-epitope constructs", *i.e.* polypeptides comprising a plurality of epitopes derived from one or more antigens, where the epitopes are organized in a non-naturally occurring order with a view to optimizing antigenicity or immunogenicity of the construct. Hence, the multi-epitope

25 expression products discussed below under the disclosure of the polynucleotides of the invention are also embodiments of the peptides of the invention.

In such multi-epitope constructs, the the plurality of epitopes is typically selected from a plurality of CTL epitopes, a plurality of B-cell epitopes, a plurality of T helper lymphocyte epitopes, a plurality of CTL and B-cell epitopes, a plurality of CTL and T helper lymphocyte

30 epitopes, a plurality of B-cell and T helper lymphocyte epitopes, and a plurality of B-cell, CTL and T helper lymphocyte epitopes. These epitopes may be derived from on single antigenic protein, but may also be derived from at least 2 different polypeptide antigens, and in turn, these may be from the same or different species (of e.g. bacteria, virus, and parasites).

In some embodiments, the pan-DR binding oligopeptide sequences can differ from the

35 original sequence by being modified by terminal-NH_2 acylation, e.g., by alkanoyl (C_1-C_{20}) or

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thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some embodiments, these modifications may provide sites for linking to a support or other molecule.

The biological activity of the peptides identified above may be assayed in a variety of
5   systems. Typically, the ability to inhibit antigen-specific T cell activation is tested. In one exemplary protocol, an excess of peptide is incubated with an antigen-presenting cell of known MHC expression, (e.g., DR1) and a T cell clone of known antigen specificity (e.g., tetanus toxin 830-843) and MHC restriction (again, DR1), and the immunogenic peptide itself (i.e., tetanus toxin 830-843). The assay culture is incubated for a sufficient time for T cell
10   proliferation, such as four days, and proliferation is then measured using standard procedures, such as pulsing with [^{3}H]-thymidine during the last 18 hours of incubation. The percent inhibition, compared to the controls which do not receive peptide, is then calculated.

The capacity of peptides to inhibit antigen presentation in an *in vitro* assay has been correlated to the capacity of the peptide to inhibit an immune response *in vivo*. *In vivo*
15   activity may be determined in animal models, for example, by administering an immunogen known to be restricted to the particular MHC molecule recognized by the peptide, and the immunomodulatory peptide. T lymphocytes are subsequently removed from the animal and cultured with a dose range of immunogen. Inhibition of stimulation is measured by conventional means, e.g., pulsing with [^{3}H]-thymidine, and comparing to appropriate
20   controls. Certain experimental details will of course be apparent to the skilled artisan. See also, Adorini, *et al., Nature* **334**: 623-625 (1988), incorporated herein by reference.

A large number of cells with defined MHC molecules, particularly MHC Class II molecules, are known and readily available from, for instance, the American Type Culture Collection (*see, e.g.,* "Catalogue of Cell Lines and Hybridomas," 6th edition (1988)) Rockville, Maryland,
25   U.S.A.

Some embodiments of the oligopeptides of the present invention comprise modifications to the N- and C-terminal residues. As will be well understood by the artisan, the N- and C-termini may be modified to alter physical or chemical properties of the peptide, such as, for example, to affect binding, stability, bioavailability, ease of linking, and the like.

30   Optionally, modifications of peptides with various amino acid mimetics or D-amino acids, for instance at the N- or C- termini, can be used, and are useful for instance, in increasing the stability of the peptides *in vivo*. Such peptides may be synthesized as "inverso" or "retroinverso" forms, that is, by replacing L-amino acids of a sequence with D-amino acids, or

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by reversing the sequence of the amino acids and replacing the L-amino acids with D-amino acids. As the D-peptides are substantially more resistant to peptidases, and therefore are more stable in serum and tissues compared to their L-peptide counterparts, the stability of D-peptides under physiological conditions may more than compensate for a difference in affinity compared to the corresponding L-peptide. Further, L-amino acid containing peptides with or without substitutions can be capped with a D-amino acid to inhibit exopeptidase destruction of the immunogenic peptide.

In vivo stability of polypeptides 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); Walter, *et al., Proc. Soc. Exp. Biol. Med.* **148**:98-103 (1975); Witter, *et al., Neuroendocrinology* **30**:377-381 (1980); Verhoef, *et al., J. Endocrinology* **110**:557-562 (1986); Handa, *et al., Eur. J. Pharmacol.* **70**:531-540 (1981); Bizzozero, *et al., Eur. J. Biochem.* **122**:251-258 (1982); Chang, *Eur. J. Biochem.* **151**:217-224 (1985), all of which are incorporated herein by reference.

The peptides or analogs of the invention can be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, *e.g.,* those at critical contact sites, may generally not be altered without an adverse effect on biological activity. The 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-protein amino acids as well, such as β-γ-δ-amino acids, as well as many derivatives of L-α-amino acids. An oligopeptide of the present invention can comprise either L-amino acids or D-amino acids, but usually not D-amino acids within a core binding region.

The peptides of the invention can be prepared in a wide variety of ways. In some embodiments, recombinant DNA technology is employed wherein a nucleotide sequence which encodes an immunogenic polypeptide of the invention 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 (1989). Alternatively, depending on their size, the polypeptides 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.*

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III.   Polynucleotides

The present invention provides for polynucleotides encoding the polypeptides of the invention
as described herein. For example, polynucleotides encoding a polypeptide comprising an
oligopeptide sequence that can bind an MHC molecule encoded by at least 2, 3, 4, 5, 6, 7, 8,
9, 10, 11, 12, or more different DR alleles, wherein the oligopeptide sequence comprises
$AX_1FVAAX_2TLX_3AA_4A$ (SEQ ID NO:1), wherein X_1 is selected from the group consisting of W, F,
Y, H, D, E, N, Q, I and K; X_2 is selected from the group consisting of F, N, Y and W; X_3 is
selected from the group consisting of H and K, and A_4 is selected from A, D, and E. In some
embodiments, the polypeptide comprises an oligopeptide sequence that can bind an MHC
molecule encoded by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more different DR alleles,
wherein the oligopeptide sequence comprises $AX_1FVAAX_2TLHAAA$ (SEQ ID NO:2), wherein X_1
is selected from the group consisting of Y, H, I, E, N, Q and K; and X_2 is selected from the
group consisting of F, N, Y and W. According to the invention, the oligopeptide sequence does
not comprise AKFVAAWTLKAAA (SEQ ID NO: 3). In some embodiments, the polynucleotides
of the present invention encode one or more amino acid sequences, each constituting the 13
amino acid C-terminal fragment of a oligopeptide selected from SEQ ID NO: 1, SEQ ID NO: 2,
SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9,
SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID
NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20,
SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID
NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31,
SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID
NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42,
SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID
NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53,
SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID
NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64,
SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID
NO: 70, SEQ ID NO: 71, or SEQ ID NO: 72; the polynucleotides may encode polypeptides
which include the sequences or truncates in fusion with other amino acid sequences. So,
polynucleotides of the invention are provided that encode polypeptides, as disclosed herein,
comprising two or more CTL and/or HTL oligopeptide sequences, including at least one pan-
DR oligopeptide sequence of the invention.

Polynucleotide constructs of the present invention can encode any of the above-described
peptides or polypeptides. For example, multiple class I and/or class II MHC epitopes and/or
B-cell epitopes, for example, more than one class II MHC epitopes (i.e., at least one pan-DR-

binding oligopeptide sequence as described herein, e.g., SEQ ID NOs: 1, 2 and 4-72) or a combination of class I MHC epitopes and class II MHC epitopes and B-cell epitopes. Class I MHC-encoding epitope nucleic acids are also referred to as "CTL epitope nucleic acids," and class II MHC-encoding epitope nucleic acids are referred to as "HTL epitope nucleic acids". "B-cell epitopes" are epitopes, either linear or conformational, which bind antibodies or B-cell receptors. Some multi-epitope constructs can have a portion of their sequence encoding class I MHC epitopes and/or B-cell epitopes and another portion encoding class II MHC epitopes. In some embodiments, the CTL epitope nucleic acids encode an epitope peptide of about eight to about thirteen amino acids in length, e.g., about eight to about eleven amino acids in length, e.g., about nine amino acids in length. The HTL epitope nucleic acids encode at least one pan-DR oligopeptide as described herein, but where more than one HTL epitope is included, other HTL epitopes can be used as well. In some embodiments, the polynucleotide constructs include, for example, five or more, ten or more, fifteen or more, twenty or more, or twenty-five or more epitope nucleic acids. All of the CTL epitope nucleic acids in a multi-epitope polynucleotide construct can be from one organism (e.g., the nucleotide sequence of every epitope nucleic acid may be present in HIV strains), or the multi-epitope construct can include epitope nucleic acids present in two or more different organisms (e.g., some epitopes from HIV and some from HCV). As described hereafter, one or more epitope nucleic acids in the multi-epitope construct may be flanked by a spacer nucleic acid.

As will be understood, nucleic acid constructs encoding B-cell epitope containing polypeptides linked to or otherwise including the PADRE analogues of the invention are an embodiment of the invention. Such nucleic acid constructs typically encode a fusion construct where the PADRE analogues present in the expression product do not interfere negatively with the conformation of B-cell epitopes from the polypeptide. In cases where the B-cell epitopes are linear, the B-cell epitopes may simply be fused to the PADRE analogues, so the provision of the encoding nucleic acid is relatively uncomplicated. When the B-cell epitopes are conformational, however, typical examples of introduction points for the PADRE analogue encoding nucleic acids are in flexible loops of the polypeptide or in flexible termini (where 3D-structure of the conformational epitopes is sought preserved, e.g. by using entire protein domains or even entire polypeptides), but it is also possible to introduce the PADRE analogue encoding nucleic acid in regions encoding e.g. intracellularly confined parts of the polypeptide in question, since these regions are not relevant for immune responses in vivo. The expression products of such nucleic acid constructs are useful as antibody inducing immunogens, because the PADRE analogue sequences introduced will provide for increased T-lymphocyte help in the elicitation of an immune response against the expression product. As such, the expression products may be used directly as immunogens (for antibody induction in e.g. a vaccine or an immunogenic composition used for antibody production in

animals) or it may exert its effect after being expressed in vivo in an animal which has been subjected to nucleic acid immunization with the encoding nucleic acid.

A "spacer" refers to a sequence that is inserted between two epitopes in a multi-epitope construct to prevent the occurrence of junctional epitopes and/or to increase the efficiency of processing. A multi-epitope construct may have one or more spacer nucleic acids. A spacer nucleic acid may flank each epitope nucleic acid in a construct, or the spacer nucleic acid to epitope nucleic acid ratio may be about 2 to 10, about 5 to 10, about 6 to 10, about 7 to 10, about 8 to 10, or about 9 to 10, where a ratio of about 8 to 10 has been determined to yield favorable results for some constructs.

The spacer nucleic acid may encode one or more amino acids. In some embodiments a spacer nucleic acid flanking a class I MHC epitope in a multi-epitope construct is between one and about eight amino acids in length, between two and eight amino acids in length, between three and eight amino acids in length, between four and eight amino acids in length, between five and eight amino acids in length, between six and eight amino acids in length, or between seven and eight amino acids in length. A spacer nucleic acid flanking a class II MHC epitope in a multi-epitope construct is in some embodiments greater than five, six, seven, or more amino acids in length, and in some embodiments greater than five or six amino acids in length.

The number of spacers in a construct, the number of amino acids encoded in a spacer polynucleotide, and the amino acid composition of a spacer can be selected to optimize epitope processing and/or minimize junctional epitopes. In some embodiments spacers are selected by concomitantly optimizing epitope processing and junctional motifs. Suitable amino acids for optimizing epitope processing are described herein. Also, suitable amino acid spacing for minimizing the number of junctional epitopes in a construct are described herein for class I and class II HLAs. For example, spacers flanking class II MHC epitopes can in some embodiments include G, P, and/or N residues as these are not generally known to be primary anchor residues (see, e.g., PCT/US00/19774). In some embodiments a spacer for flanking a class II MHC epitope includes alternating G and P residues, for example, $(GP)_n$, $(PG)_n$, $(GP)_nG$, $(PG)_nP$, and so forth, where n is an integer between one and ten, between two or about two, and in some embodiments a specific example of such a spacer is GPGPG or PGPGP. In some embodiment for class I MHC epitopes, the spacer comprises one, two, three or more consecutive alanine (A) residues, optionally preceded by K, N or G.

In some multi-epitope constructs, it is sufficient that each spacer nucleic acid encode the same amino acid sequence. In multi-epitope constructs having two spacer nucleic acids

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encoding the same amino acid sequence, the spacer nucleic acids encoding those spacers may have the same or different nucleotide sequences, where different nucleotide sequences may decrease the likelihood of unintended recombination events when the multi-epitope construct is inserted into cells.

5   In other multi-epitope constructs, one or more of the spacer nucleic acids may encode different amino acid sequences. While many of the spacer nucleic acids may encode the same amino acid sequence in a multi-epitope construct, one, two, three, four, five or more spacer nucleic acids may encode different amino acid sequences, and it is possible that all of the spacer nucleic acids in a multi-epitope construct encode different amino acid sequences.
10   Spacer nucleic acids may be optimized with respect to the epitope nucleic acids they flank by determining whether a spacer sequence will maximize epitope processing and/or minimize junctional epitopes, as described herein.

Multi-epitope constructs may be distinguished from one another according to whether the spacers in one construct optimize epitope processing or minimize junctional epitopes over
15   another construct, and in some embodiments, constructs may be distinguished where one construct is concomitantly optimized for epitope processing and junctional epitopes over the other. Computer assisted methods and *in vitro* and *in vivo* laboratory methods for determining whether a construct is optimized for epitope processing and junctional motifs are described herein.

20   In some embodiments, polynucleotide constructs of the invention are provided as an expression vector comprising a nucleic acid encoding at least one pan-DR oligopeptide sequence of the invention, and optionally any or all of the sequences described herein in the context of polypeptides. Construction of such expression vectors is described, for example in PCT/US99/10646, the disclosure of which is herein incorporated by reference. The expression
25   vectors contain at least one promoter element that is capable of expressing a transcription unit encoding the nucleic acid in the appropriate cells of an organism so that the antigen is expressed and targeted to the appropriate MHC molecule. For example, for administration to a human, a promoter element that functions in a human cell is incorporated into the expression vector.

30   Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2nd ed. 1989); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel *et al.*, eds., 1994); Oligonucleotide Synthesis. A Practical Approach (Gait, ed., 1984); Kuijpers, *Nucleic Acids Research 18(17)*:5197 (1994); Dueholm, *J. Org. Chem. 59*:5767-5773

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(1994); Methods in Molecular Biology, volume 20 (Agrawal, ed.); and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, e.g., Part I, chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" (1993)).

5   The nucleic acids encoding the relevant oligopeptide sequences (e.g., epitopes) can be assembled in a construct according to standard techniques. In some embodiments, the nucleic acid sequences encoding pan-DR binding oligopeptides, and optionally multi-epitope polypeptides, are isolated using amplification techniques with oligonucleotide primers, or are chemically synthesized. Recombinant cloning techniques can also be used when appropriate.

10   Oligonucleotide sequences are selected which either amplify (when using PCR to assemble the construct) or encode (when using synthetic oligonucleotides to assemble the construct) the desired epitopes.

Amplification techniques using primers are typically used to amplify and isolate sequences encoding the epitopes of choice from DNA or RNA (see U.S. Pat. Nos. 4,683,195 and

15   4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis et al., eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify epitope nucleic acid sequences directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Restriction endonuclease sites can be incorporated into the primers. Multi-epitope constructs amplified by the PCR reaction can be purified from

20   agarose gels and cloned into an appropriate vector.

Synthetic oligonucleotides can also be used to construct the polynucleotides of the invention. In some embodiments, this method is performed using a series of overlapping oligonucleotides, representing both the sense and non-sense strands of the gene. These DNA fragments are then annealed, ligated and cloned. Oligonucleotides that are not commercially

25   available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts. 22*:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al., Nucleic Acids Res., 12*:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.*

30   *255*:137-149 (1983).

In some embodiments, the epitopes of polynucleotide constructs of the invention are subcloned into an expression vector that contains a strong promoter to direct transcription, as well as other regulatory sequences such as enhancers and polyadenylation sites. Suitable promoters are well known in the art and described, e.g., in Sambrook et al. and Ausubel et

al. Eukaryotic expression systems for mammalian cells are well known in the art and are commercially available. Such promoter elements include, for example, cytomegalovirus (CMV), Rous sarcoma virus LTR and SV40.

The expression vector typically contains a transcription unit or expression cassette that
5   contains all the additional elements required for the expression of the polynucleotide construct in host cells. For example, an expression cassette can contain a promoter operably linked to a multi-epitope construct and signals required for efficient polyadenylation of the transcript. Additional elements of the cassette may include enhancers and introns with functional splice donor and acceptor sites.

10   In addition to a promoter sequence, the expression cassette can also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

Selectable markers can be incorporated into the expression vectors used to express the
15   peptides of the invention. These genes can encode a gene product, such as a protein, necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics or other toxins, such as ampicillin, neomycin, kanamycin, chloramphenicol, or
20   tetracycline. Alternatively, selectable markers may encode proteins that complement auxotrophic deficiencies or supply critical nutrients not available from complex media. In some embodiments, the vector will have one selectable marker that is functional in, *e.g.*, *E. coli*, or other cells in which the vector is replicated prior to being introduced into the host cell. A number of selectable markers are known to those of skill in the art.

25   A variety of expression vectors can be used to transport the genetic information into the cell. Any of the conventional vectors used for expression in eukaryotic cells may be used. Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, CMV vectors, papilloma virus vectors, and vectors derived from Epstein Bar virus.

30   The polynucleotide constructs of the invention can be expressed from a variety of vectors including plasmid vectors as well as viral or bacterial vectors. Examples of viral expression vectors include attenuated viral hosts, such as vaccinia or fowlpox. As an example of this approach, vaccinia virus is used as a vector to express nucleotide sequences that encode the

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(poly)peptides of the invention. Upon introduction into a host bearing a tumor, 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. Pat. No. 4,722,848.

5   The polynucleotides of the invention can be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast and insect cells, so as to obtain the protein expression products described herein. The host cells can be microorganisms, such as, for example, yeast cells, bacterial cells, or filamentous fungal cells. Examples of suitable host cells include, for example, *Pseudomonas sp.*, *Escherichia sp.* (*e.g.*, *E. coli*), *Bacillus,* among many others.
10   Suitable yeast cells can be of any of several genera, including, for example, *Saccharomyces* (*e.g.*, *S. cerevisiae*), and *Candida*. Suitable fungal cells are selected from Pichia spp., Aspergillus spp, and other fungi suitable for recombinant production. Suitable insect cells can be of several varieties, including, for example, S2, Sf9 and Hi-5 cells.

     A wide variety of other vectors useful for therapeutic administration or immunization, e.g.
15   adeno and adeno-associated virus vectors, retroviral vectors, non-viral vectors such as BCG (Bacille Calmette Guerin), Salmonella typhi vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art. So, the invention also relates to prophylactic or therapeutic use of such vectors by means of administration to an individual in need thereof of such vectors.

20   Immunogenicity and antigenicity of the multi-epitope constructs are evaluated as described herein.

     As set forth above, the present invention also relates to isolated polypeptides encoded by any of the polynucleotides of the invention as described herein – as such, these polypeptides are also "peptides" or "polypeptides" of the present invention.

25   IV.   Further Antigens

     As explained above, the pan-DR oligopeptides of the invention can be used to enhance a cellular or humoral immune response to a variety of antigens. Antigenic determinants from such an antigen, or the antigen as such, can be administered admixed with or linked to the pan-DR binding peptides of the present invention. For example, the antigenic determinant
30   can be linked or mixed, or administered in series, with the helper peptides of the present invention to elicit or enhance an immune response. Essentially any antigen can be used (e.g., polysaccharides, proteins, glycoproteins, lipids, glycolipids, lipopolysaccharides and the like)

in combination with the pan-DR binding oligopeptide sequences of the present invention. Protein antigens may be derived from infectious agents such as bacteria, fungi, virus, protozoans, helminths and other parasites, but they may also be disease-associated antigens, such as cancer antigens or antigens which are overexpressed or otherwise inexpedient in

5   certain disease states. The cancer antigens listed in WO 00/20027 are all relevant protein antigens, and further antigens are those associated with inflammation (here TNF is a good example). The present invention discloses a number of TNF constructs which are all part of the invention – generally, all these constructs are also part of the invention, as are nucleic acid fragments encoding them, vectors comprising the nucleic acid fragments and host cells

10   including the nucleic acid fragments or vectors (or being transformed with the vectors).

In some embodiments the oligopeptides of the present invention are administered alone. In some embodiments the oligopeptides of the invention are administered in conjunction with a second antigenic determinant. In some embodiments the oligopeptides of the invention are admixed with the antigenic determinant. In some embodiments the peptides of the invention

15   are linked to the antigenic determinant. For example, protein antigens can be linked directly or indirectly to the pan-DR binding oligopeptides as fusion proteins (via peptide bonding). Antigens, including non-protein antigens, can be linked to the pan-DR oligopeptides, or polypeptides comprising the oligopeptide sequences, via other covalent conjugation methods.

In some embodiments the antigenic determinant administered with the peptides of the

20   present invention is a protein. In some embodiments the antigenic determinant administered with the peptides of the present invention is a polysaccharide. In some embodiments the antigenic determinant administered with the peptides of the present invention is a glycoprotein. In some embodiments the antigenic determinant administered with the peptides of the present invention is a lipid. In some embodiments the antigenic determinant

25   administered with the peptides of the present invention is a glycolipid. In some embodiments the antigenic determinant administered with the peptides of the present invention is a lipopolysaccharide.

Carbohydrate epitopes include a carbohydrate structure, but can be present as a glycoconjugate, e.g., glycoprotein, glycopeptide, glycolipid, and the like, DNA, RNA, or a

30   polysaccharide, oligosaccharide, or monosaccharide against which an immune response is desired. The carbohydrate epitope may induce a wide range of immune responses. One of skill will recognize that various carbohydrate structures exemplified herein can be variously modified according to standard methods, without adversely affecting antigenicity. For instance, the monosaccharide units of the saccharide may be variously substituted or even

35   replaced with small organic molecules, which serve as mimetics for the monosaccharide.

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Examples of suitable antigens include those derived from bacterial surface polysaccharides which can be used in carbohydrate-based vaccines. Bacteria typically express carbohydrates on their cell surface as part of glycoproteins, glycoplipids, O-specific side chains of lipopolysaccharides, capsular polysaccharides and the like. Exemplary bacterial strains include

5   *Streptococcus pneumonia* (*see, e.g.,* WO/2005/120563 and carbohydrate epitopes therein), *Neisseria meningitidis, Haemophilus influenza, Klebsiella spp., Pseudomonas spp., Salmonella spp., Shigella spp.,* and Group B *streptococci.*

A number of suitable bacterial carbohydrate epitopes are described in the prior art (*e.g.,* Sanders, *et al. Pediatr. Res.* **37**:812-819 (1995); Bartoloni, *et al. Vaccine* **13**:463-470

10 (1995); Pirofski, *et al., Infect. Immun.* **63**:2906-2911 (1995) and International Publication No. WO 93/21948) and as described in, e.g., US Patent No. 6,413,935.

In general, the HLA binding epitopes disclosed in any one of the following international patent applications may according to the invention be useful and the contents of which are therefore incorporated by reference herein: WO 93/03764, WO 95/22317, WO 94/03205, WO

15 95/19783, WO 97/34617, WO 02/20053, WO 94/20127, WO 97/34621, WO 02/20616, WO 95/07707, WO 95/04817, WO 98/33888, WO 94/26774, WO 96/03140, WO 02/20035, WO 96/40213, WO 97/26784, WO 97/33602, WO 98/32456, WO 99/61916, WO 99/45954, WO 99/65522, WO 01/62776, WO 01/00225, WO 2004/031211, WO 99/58658, WO 2005/012502, WO 00/44775, WO 02/019986, WO 2004/031210, WO 01/21189, WO

20 01/24810, WO 2005/033265, WO01/42270, WO01/41788, WO01/42267, WO 01/45728, WO 0141787, WO 02/061435, WO 02/061435, WO 01/41741, WO 2004/052917, WO 01/36452, WO 03/087126, WO 01/47541, WO 02/083714, WO 01/41799, WO 2005/089164, WO 2003/040165, WO 2005/120563, WO 2004/094454, WO 2004/053086, WO 2004/089973, WO 2008/054540, WO 2008/039267, WO 99/19478, WO 92/21033, WO 94/11738, WO

25 93/22338, WO 95/25530, WO 95/25739, WO 2005/118626, WO 93/03753, WO 94/19011, WO 95/03777, WO 95/19783, WO 95/04542, WO 01/42270, WO 01/41788, WO 01/42267, WO 01/45728, WO 01/41787, WO 01/41741, WO 2004/052917, WO2004/094454, and WO 2004/089973.

V.   Preparation of Conjugates

30 The pan-DR binding peptides of the invention can be linked to at least one further antigenic determinant in a variety of ways. Ionic interactions are possible through the termini or through the ε- amino group of lysine. Hydrogen bonding between the side groups of the residues and the antigenic determinants are also possible. In other embodiments,

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conformation interactions between the pan-DR binding peptide and the antigenic determinant may give rise to a stable attachment.

As noted above, antigenic determinants may be covalently linked to the pan-DR binding peptides to prepare conjugates of the invention. In some embodiments antigenic

5   determinant/ pan-DR binding peptide conjugates are linked by a spacer molecule or linker. In some embodiments, the antigenic determinant may be attached to the pan-DR binding peptide without a linker.

The spacer or linker is typically comprised of neutral molecules, such as, aliphatic carbon chains, amino acids or amino acid mimetics, which are substantially uncharged under

10   physiological conditions and may have linear or branched side chains. A number of compositions and methods for linking various biomolecules are known to those of skill in the art. A number of methods for covalently linking a pan-DR binding peptide, for instance, to a carbohydrate epitope are possible. Methods suitable for linking pan-DR binding peptides to carbohydrate antigens are disclosed for instance in WO 93/21948.

15   A number of linkers are well known and are either commercially available or are described in the scientific literature. The linking molecules used in the present invention are of sufficient length to permit the two portions of the molecule to interact independently and freely with molecules exposed to them. In the case of carbohydrate epitopes, the linking molecules are typically 1-50 atoms long. In some embodiments, the linking molecules will be aryl acetylene,

20   ethylene glycol oligomers containing 2-14 monomer units, diamines, diacids, amino acids, or combinations thereof. Other suitable linkers include lipid molecules such as ceramide and amino acid residues to which a different carbohydrate moiety is linked through the amino acid side chain.

The particular linking molecule used may be selected based upon its chemical/physical

25   properties. The linking molecule has an appropriate functional group at each end, one group appropriate for attachment to the reactive sites on the carbohydrate portion and the other group appropriate for attachment to the amino acid/peptide portion. For example, groups appropriate for attachment to the carbohydrate portion are carboxylic acid, ester, isocyanate, alkyl halide, acyl halide and isothiocyanate. Similar groups would be useful for attachment to

30   the amino acid portion. Appropriate selection of the functional group will depend on the nature of the reactive portion of the amino acid or peptide.

In one group of embodiments, alkyl or alkylene groups will be useful as linking groups and will have 1 to 20 carbon atoms, in some embodiments contain between 3 to 6 carbon atoms.

For instance, linkers comprising polyethylene glycol and related structures can be used. The term "polyethylene glycol" is used to refer to those molecules which have repeating units of ethylene glycol, for example, hexaethylene glycol ($HO-(CH_2CH_2O)_5-CH_2CH_2OH$). When the term "polyethylene glycol" is used to refer to linking groups, it would be understood by one of

5   skill in the art that other polyethers or polyols could be used as well (i.e, polypropylene glycol or mixtures of ethylene and propylene glycols).

In another group of embodiments, the alkyl or alkylene linking groups will be perfluorinated, rendering them less susceptible to biological degradation. *see,* U.S. Patent No. 5,055,562. In some embodiments linking groups will include aminocaproic acid, 4-hydroxy butyric acid,

10   4-mercapto butyric acid, 3-amino-1-propanol, ethanolamine, perfluoroethanolamine, and perfluorohydroxybutyric acid. In some embodiments, the two portions are linked via a polyethylene glycol moiety.

In some embodiments, the linkers between pan-DR binding peptides and other peptides (for example but not limited to, a pan-DR binding peptide and a CTL or B-cell epitope) can be

15   selected from Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In some embodiments herein the neutral spacer is Ala. 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. In some embodiments, exemplary spacers are homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues,

20   more usually three to six residues. In some embodiments the pan-DR binding peptide is conjugated to the CTL or antibody- inducing peptide. In some embodiments, the pan-DR binding peptide is positioned at the amino terminus. The peptides can be joined by a neutral linker, such as Ala-Ala-Ala or the like, and in some embodiments can contain a lipid residue such as palmitic acid or the like which is attached to alpha and epsilon amino groups of a Lys

25   residue (($PAM)_2Lys$), which is attached to the amino terminus of the peptide conjugate, typically via Ser-Ser linkage or the like.

The CTL or antibody-inducing peptide may be linked to the pan-DR binding peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the CTL or antibody inducing peptide or the pan-DR binding peptide

30   can be acylated. In some embodiments, the CTL peptide/pan DR binding peptide conjugate can be linked to certain alkanoyl (C_1-C_{20}) lipids via one or more linking residues such as Gly, Gly-Gly, Ser, Ser-Ser as described below. In some embodiments lipid moieties include cholesterol, fatty acids, and the like.

In some embodiments the pharmaceutical compositions of the invention can contain at least one component which assists in priming CTL. Lipids have been identified as agents capable of assisting the priming CTL *in vivo* against viral antigens. For example but not limited to, steroids such as cholesterol, fatty acids such as palmitic acid residues can be attached to the

5   sulfhydryl group of a cysteine residue, 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, such as a pan-DR binding peptide. In some embodiments, in place of fatty acids, long chain alkyl groups can be linked through an ether linkage to the final amino acid *(e.g.,* a cysteine residue).

10   The lipidated peptide can be injected, either directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, *e.g.,* incomplete Freund's adjuvant. In some embodiments 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.

15   As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinlyseryl-serine (P$_3$CSS) can be used to prime virus specific 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$_3$CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. In

20   some embodiments, the induction of neutralizing antibodies can also be primed with P$_3$CSS conjugated to a peptide which displays an appropriate epitope and the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

In the case of pan-DR binding peptides conjugated to carbohydrate epitopes, the lipid moieties may be linked to the opposite terminus of the peptide *(e.g.,* carbohydrate linked to

25   the C-terminus and lipid linked to the N-terminus). In some embodiments, both the lipid and the carbohydrate moieties may be linked to the same end of the peptide. In some embodiments, the two moieties may be linked to the same linker on the N-terminus.

VI.   Methods of stimulating an immune response

Polypeptide immunogens of the present invention may be expressed, concentrated and

30   purified from expression hosts such as *E. coli* (or other host cells discussed above) using various methods known to one of skill in the art – alternatively, the immunogens may be prepared by liquid or solid phase peptide synthesis. Purified polypeptide or peptide immunogens can be formulated with pharmaceutically-acceptable excipients for

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administration into individuals, for example, to stimulate or enhance an immune response
and optionally generate a protective and/or therapeutic immune response. The phrase
"protective and/or therapeutic immune response" refers to a CTL and/or an HTL and/or
antibody response to a disease related antigen, e.g. derived from an infectious agent, which
in some way prevents or at least partially arrests disease symptoms, side effects or
progression, and clears the infectious agent. In some embodiments, the polypeptides or
polynucleotides of the invention are formulated into vaccines containing an immunologically
effective amount of one or more of the peptides of the invention and an appropriate
pharmaceutical carrier. Thus, peptides of the invention can be administered individually or in
combination either in a single composition or multiple compositions.

The invention further relates to methods of administering a pharmaceutical composition
comprising an expression vector of the invention or a polypeptide derived therefrom to
stimulate an immune response. The expression vectors are administered by methods well
known in the art as described in, for example, Donnelly *et al.* (*Ann. Rev. Immunol.*, *15*:617-
648 (1997)); Felgner *et al.* (U.S. Pat. No. 5,580,859, issued Dec. 3, 1996); Felgner (U.S. Pat.
No. 5,703,055, issued Dec. 30, 1997); and Carson *et al.* (U.S. Pat. No. 5,679,647, issued
Oct. 21, 1997). In one embodiment, the multi-epitope construct is administered as naked
nucleic acid.

A pharmaceutical composition comprising an expression vector of the invention or a
polypeptide derived therefrom can be administered to stimulate an immune response in a
subject by various routes including, for example, orally, intravaginally, rectally, or
parenterally, such as intravenously, intramuscularly, subcutaneously, intraorbitally,
intracapsularly, intraperitoneally, intracisternally or by passive or facilitated absorption
through the skin using, for example, a skin patch or transdermal iontophoresis, respectively.
Furthermore, the composition can be administered by injection, intubation or topically, the
latter of which can be passive, for example, by direct application of an ointment or powder, or
active, for example, using a nasal spray or inhalant. An expression vector also can be
administered as a topical spray, in which case one component of the composition is an
appropriate propellant. The pharmaceutical composition also can be incorporated, if desired,
into liposomes, microspheres or other polymer matrices as described in, for example, Felgner
et al., U.S. Pat. No. 5,703,055; Gregoriadis, Liposome Technology, Vols. I to III (2nd ed.
1993). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic,
physiologically acceptable and metabolizable carriers that are relatively simple to make and
administer.

The expression vectors of the invention or a polypeptide derived therefrom can be delivered to the interstitial spaces of tissues of an animal body as described in, for example, Felgner *et al.*, U.S. Pat. Nos. 5,580,859 and 5,703,055. Administration of expression vectors of the invention to muscle is a particularly effective method of administration, including intradermal and subcutaneous injections and transdermal administration. Transdermal administration, such as by iontophoresis, is also an effective method to deliver expression vectors of the invention to muscle. Epidermal administration of expression vectors of the invention can also be employed. Epidermal administration involves mechanically or chemically irritating the outermost layer of epidermis to stimulate an immune response to the irritant (Carson *et al.*, U.S. Pat. No. 5,679,647).

Other effective methods of administering an expression vector of the invention or a polypeptide derived therefrom to stimulate an immune response include mucosal administration as described in, for example, Carson *et al.*, U.S. Pat. No. 5,679,647. For mucosal administration, the most effective method of administration includes intranasal administration of an appropriate aerosol containing the expression vector and a pharmaceutical composition. Suppositories and topical preparations are also effective for delivery of expression vectors to mucosal tissues of genital, vaginal and ocular sites. Additionally, expression vectors can be complexed to particles and administered by a vaccine gun.

To conclude, the peptides of the present invention are useful for inducing immune responses, both with a view to prophylaxis (*i.e.* with a view to reducing risk of later disease) and with a view to treatment – however the peptides of the invention are also useful for inducing immune responses experimentally and with a view to inducing antibodies in animals from which antibodies and/or B lymphocytes can be isolated – this in turn allows for later production of monoclonal antibodies and antibody derivatives.

VII.   Pharmaceutical compositions

The polypeptides of the present invention, polynucleotides of the invention, and pharmaceutical and vaccine compositions thereof, can be administered to mammals, particularly humans, for prophylactic and/or therapeutic purposes. The polypeptides of the present invention can be used to elicit and/or enhance immune responses against antigens, including but not limited to, pathogen or cancer-associated or cancer-specific biomolecules (e.g., proteins, carbohydrates, etc.). Examples of diseases which can be treated using the present invention include various bacterial infections, viral infections, fungal infections, parasitic infections and cancer. As discussed above with respect to proteinaceous antigens,

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the cancers associated with the cancer antigens from WO 00/20027 are also examples of diseases as are various inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

In some therapeutic applications, the present invention is administered to an individual already suffering from cancer, inflammatory diseases or infected with the virus or microorganism of interest. Those in the incubation phase or the acute phase of the disease may be treated with the present invention separately or in conjunction with other treatments, as appropriate.

In some therapeutic applications, a composition of the present invention is administered to a patient in an amount sufficient to elicit an effective CTL response or humoral response to the microorganism or 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." Amounts effective for this use will depend in part on 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.

Therapeutically effective amounts of the compositions of the present invention generally range for the initial immunization that is for therapeutic or prophylactic administration, from about 1.0 μg to about 10,000 μg of polypeptide for a 70 kg patient, e.g., from about 100 to about 8000 μg, e.g., between about 200 and about 6000 μg. In some embodiments, these doses are followed by boosting dosages of from about 1.0 μg to about 3000 μg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific immune responses.

It should be kept in mind that the compositions of the present invention can 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 conjugates, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these compositions.

In some embodiments, the present invention can be used prophylactically to prevent and/or ameliorate a particular disease, including but not limited to, bacterial infections, viral infections, fungal infections, parasitic infections and cancer. Effective amounts are as described above. Additionally, one of ordinary skill in the vaccine arts would also know how to adjust or modify prophylactic treatments, as appropriate, for example by boosting and adjusting dosages and dosing regimes.

Formulated vaccines of the invention can be combined with a pharmaceutically acceptable adjuvant. The formulated vaccines can be an aqueous solution, a suspension or an emulsion. An immunologically effective amount of each immunogen in the vaccines of the present invention is determinable by methods known in the art without undue experimentation.

5   The adjuvant can be any pharmaceutically acceptable adjuvant. In some embodiments the adjuvant is an alum based compound. In some embodiments the adjuvant is aluminum hydroxide. In others the adjuvant is aluminum phosphate. In some embodiments the adjuvant is Emunade®. Emunade® is an adjuvant consisting of a combination of oil, water and aluminum hydroxide. In some embodiments the adjuvant is Quil A. In some
10   embodiments the adjuvant is Quil A plus cholesterol. In some embodiments the adjuvant is an emulsion, such as but not limited to MF59 and Provax.

In some embodiments, ISCOM is used as an adjuvant. ISCOM is an acronym for Immune Stimulating Complex and the technology is described, e.g., in Morein *et al.* (*Nature* 308:457-460 (1984)). ISCOMs are lipophilic immune stimulating complexes formed as follows. The
15   polypeptides are solubilized using standard methods, such as with a non-ionic detergent (e.g., Mega-9, Triton X-100, Octylglucoside, Digitonin, Nonidet P-40, $C_{12}E_8$, Lubrol, Tween-80). A lipid mixture is added to assist ISCOM formation. The lipid mixture can include a phosphatidyl choline and a synthetic cholesterol. In some embodiments, the mixture is first treated with non-ionic detergent at room temperature with stirring, then the lipid mixture
20   (equal parts phosphatidyl choline and cholesterol, for example) is added and stirring continued. Quil A (a purified glycoside of saponin) is added to polypeptide composition and stirring is continued. Then the non-ionic detergent is removed (for example, by diafiltration with ammonium acetate). The matrix of the ISCOM is formed by Quil A. The morphology of an ISCOM particle, as viewed by electron microscopy, shows a typical cage like structure of
25   approximately 35 nm in size. The ISCOM formation stage can be refined by the use of tangential flow diafiltration. ISCOMs present purified antigens in a multimeric form based on the ability of Quil A to spontaneously form micelles at a critical concentration and by a hydrophobic/hydophilic link that entrap the purified antigens. Formation of ISCOMs can be verified by electron microscopy to verify that the typical cage-like structures have been
30   formed. The Quil A can be added to give a final concentration of about 0.01 to 0.1 %. In some embodiments, the final concentration is about 0.05%.

The invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and an expression vector of the invention or a polypeptide of the invention. Pharmaceutically acceptable carriers are well known in the art and include aqueous or non-
35   aqueous solutions, suspensions and emulsions, including physiologically buffered saline,

alcohol/aqueous solutions or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters, lipids or liposomes.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize the expression vector or increase the absorption of the

5   expression vector. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants such as ascorbic acid or glutathione, chelating agents, low molecular weight polypeptides, antimicrobial agents, inert gases or other stabilizers or excipients. Expression vectors can additionally be complexed with other components such as peptides, polypeptides and carbohydrates. Expression vectors

10   can also be complexed to particles or beads that can be administered to an individual, for example, using a vaccine gun. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector.

The invention further relates to methods of administering a pharmaceutical composition

15   comprising an expression vector of the invention or a polypeptide derived therefrom to stimulate an immune response. The expression vectors are administered by methods well known in the art as described in, for example, Donnelly *et al.* (*Ann. Rev. Immunol., 15*:617- 648 (1997)); Felgner *et al.* (U.S. Pat. No. 5,580,859, issued Dec. 3, 1996); Felgner (U.S. Pat. No. 5,703,055, issued Dec. 30, 1997); and Carson *et al.* (U.S. Pat. No. 5,679,647, issued

20   Oct. 21, 1997). In one embodiment, the polynucleotides of the invention are administered as naked nucleic acid.

The compositions of the invention are also useful for induction of immune responses, e.g. in experimental animals or in animals with a view to prepare antibodies or antibody derivatives. In such compositions the requirement that the composition should be pharmaceutically

25   acceptable is of minor importance, and it is e.g. possible to use adjuvants not considered suitable for use in humans.

PREAMBLE TO EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Proteolytic cleavage within PADRE (SEQ ID NO: 3) has been observed in PADRE-containing

30   proteins, in particular around the two lysine residues K2 and K10 but also around W7. Cleavage within PADRE is undesirable because it, in recombinantly produced proteins, leads

to heterogeneity, reduced production yield and altered stability. Even if cleavage can be successfully avoided by the use of additives during upstream and downstream processes, a resulting PADRE containing vaccine may still have poor immunogenicity and pharmacokinetics properties such as reduced half-life and improper presentation of the T helper epitope(s).

5   Thus there is a need to remove protease sensitive amino acids / sequences from the PADRE sequence.

Provision of PADRE analogues having reduced susceptibility to protease activity will reduce the susceptibility of recombinant expression products to proteases especially in expression systems such as *E. coli* and *Drosophila* expression systems in order to avoid proteolytic

10   cleavages within the pan DR binding peptide amino acid sequence during up-stream and/or downstream processes of protein production. Further, in the event a PADRE analogue is administered to humans, either in isolated form or as part of an immunogenic polypeptide, the reduced susceptibility will entail the further advantage of improved pharmacokinetic properties (i.a. longer serum half-life) and possibly improved immunogenicity.

15   On this basis a number of analogues of SEQ ID NO: 3 have been prepared and subjected to the following sequence of identification steps in order to identify improved pan DR binding peptides which are less prone to proteolytic degradation:

1) Preparation of library of synthetic PADRE analogues
2) Assaying and selecting analogues from 1) for good HLA binding properties
20   3) Assaying and selecting analogues selected in 2) for *in situ* protease resistance
4) Assaying and selecting analogues selected in 3) for good immunogenic properties.

EXAMPLE 1
Testing for HLA-DR binding of PADRE analogues

A total of 69 different PADRE analogues (SEQ ID NOs: 4-72) were synthesized by standard

25   solid phase peptide synthesis - all these PADRE analogues are constituted by, from the N- to the C-terminus, two alanine residues followed by residues 3-15, which is an analogue of SEQ ID NO: 3 (except for SEQ ID NO: 4, which is PADRE preceded by two alanine residues). The reason for including the 2 extra N-terminal alanines was to ensure that the isolated peptides would be able to demonstrate endoprotease resistance in an *in vitro* assay for protease

30   resistance, whereas the immunogenic properties of the PADRE analogues reside in the 13 C-terminal amino acids.

The peptides were tested for binding to HLA-DR according to the following procedure:

Peptide/HLA-DR binding assays

The standard operating procedure for the class II MHC binding assay is described in Sidney *et al.* (1998), Current Protocols in Immunology, 18.3.1 - 18.3.19, 1998. Briefly, purified human class II molecules [5 to 500nM] were incubated with various unlabeled peptide binding inhibitors and 1-10nM ^{125}I-radiolabeled probe peptides for 48h in PBS containing 5% DMSO in the presence of a protease inhibitor cocktail. Final detergent concentration in the incubation mixture was 0.05% Nonidet P-40. Assays were performed at pH 7.0 with the exception of DR3, which was performed at pH 4.5, and DRw53, which was performed at pH 5.0. The pH was adjusted as described in Sette *et al.*(1992), J Immunol, 148:844-51.

Instead of using HPLC methodology to measure peptide binding to MHC molecules as detailed in Sidney et al., an anti-MHC class II antibody coated plate-based capture assay was utilized. This assay has been developed to use a 96-well white polystyrene microtiter plate specifically designed for high-volume, in-plate, radiometric assays. Measurement of the ^{125}I-labeled peptide bound to MHC is accomplished using the TOPCOUNT (Perkin-Elmer Instruments) benchtop microplate scintillation and luminescence counter, which allows for a highly sensitive assay for high throughput performance.

Peptide binding inhibitors were tested at concentrations ranging from 30 μg/ml to 300 pg/ml. Utilizing commercially available curve-fitting algorithms, the data were plotted *in silico* and the dose yielding 50% inhibition (IC_{50}) was determined. In appropriate stoichiometric conditions, the IC_{50} of an unlabeled test peptide to the purified DR molecule is a reasonable approximation of the affinity of interaction (K_D). Peptides were tested in two to four completely independent experiments.

Results

The binding affinities as indicated via the IC_{50} values are listed for each peptide in Fig. 1. On the basis of these IC_{50} values, it was concluded that a fraction of the analogues (38 PADRE analogues) exhibited sufficiently good HLA binding capabilities (shown in Fig. 1 by bold and underlining). Of these good binders, 10 were initially selected for further testing for protease resistance: SEQ ID NOs: 4, 10, 19, 23, 29, 38, 46, 52, 67, and 69.

It is also important to note that the inclusion of the two N-terminal alanines in SEQ ID NOs. 4-69 did not affect the HLA binding properties as evidenced by the similar binding characteristics exhibited by SEQ ID NOs: 3 and 4.

EXAMPLE 2

Protease resistance testing of selected PADRE analogues

In order to test the protease resistance of selected, good binding PADRE analogues, their
sequences were inserted by cloning techniques into a loop (loop EF) in human TNFα. Cell-free
5   protein crude extract from *E. coli* expressing the modified TNFα molecules was prepared and
proteolytic cleavage within the PADRE analogue sequences was analysed by SDS-PAGE
followed by immunoblotting.

Full-length, membrane-bound TNFα is a 233 amino-acid long protein. The amino-terminal
fragment 1-76 is absent in the 157 amino-acid long soluble form of TNFα (77-233).

10   The template used for the design of human TNFα vaccine candidates is the soluble form of
TNFα (fragment 77-233, SEQ ID NO: 73), preceded by an N-terminal Met for expression in *E.
coli*. Furthermore a point mutation was made (Y87S) in order to abolish binding of TNFα to its
two receptors, thereby abolishing the cytotoxicity of the protein. Thus, the vaccine candidates
are based on the 158 amino acid residue long SEQ ID NO: 74:

15   TNF 37.87 (SEQ ID NO: 75) is a TNF variant where PADRE is inserted between A_{185} and E_{186}
(...PEGA-EAK...). Since PADRE begins with an A, the A was not duplicated, resulting in an
insertion of 12 amino acids instead of 13:

...PEGA-AKFVAAWTLKAAA-EAK... ➜ PEG-**A-**KFVAAWTLKAAAEAK...

The TNF variants initially tested for protease resistance are thus

20   SEQ ID NO: 75 - TNF 37.87 – reference protein comprising "traditional" PADRE:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEG<u>AKFVAAWTLKAAA</u>EAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

- as well as 9 PADRE analogue containing TNF variants, each similarly having the 12 C-
25   terminal amino acid residues of SEQ ID NOs: 4, 10, 19, 23, 29, 38, 46, 52, 67, and 69,
respectively, at the underlined position:

SEQ ID NO: 76 - TNF 37.87-**007** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG

35

CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>W FVAAN TLH AAA</u>EAKPWYEPIYLGGVFQLE
KGDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 77 - TNF 37.87-**016** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>FFVAAN TLKAD</u>AEAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 78 - TNF 37.87-**020** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>YFVAAFTLH AAA</u>EAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 79 - TNF 37.87-**026** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>H FVAAN TLH AAA</u>EAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 80 - TNF 37.87-**035** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>H FVAAFTLKAE</u>AEAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 81 - TNF 37.87-**043** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>EFVAAWTLH AAA</u>EAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 82 - TNF 37.87-**049** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>N FVAAYTLH AAA</u>EAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 83 - TNF 37.87-**064** sequence:
<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEGA<u>QFVAAN TLH AAA</u>EAKPWYEPIYLGGVFQLE
KGDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 84 - TNF 37.87-**066** sequence:

<u>M</u>VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQG
CPSTHVLLTHTISRIAVS<u>S</u>QTKVNLLSAIKSPCQRETPEG<u>AI FVAAWTLH</u>AAAEAKPWYEPIYLGGVFQLEK
GDRLSAEINRPDYLDFAESGQVYFGIIAL

5   In all sequences, double underlining denotes the initial methionyl and the Y87S mutation,
respectively, and single underlining denotes the pan DR binding sequence inserted; bold
indicates amino acid changes in the pan DR binding peptide relative to PADRE (SEQ ID NO:
3).

<u>Cloning, expression, purification and characterization of recombinant TNFα variants</u>

10   A synthetic cDNA encoding TNF (Y87S) was used as template. Oligonucleotides encoding
PADRE or PADRE analogues were inserted into the template between A_{185} and E_{186} by PCR (cf.
above). Resulting cDNAs were cloned into pET28b+ (Novagen) and transformed into *E. coli*
strain HMS174. For recombinant expression, the resulting *E. coli* strains were cultured in
fermentors in a defined medium at 37°C. Induction of recombinant expression was initiated
15   by the addition of 1 mM of IPTG and temperature was lowered to 25°C. Cultures were
harvested at 4, 8 or 24h post-IPTG induction. TNFα variants were purified to > 90%
homogeneity by affinity chromatography using a TNFα specific monoclonal antibody as
described in Nielsen *et al.*, (2004), J Biol. Chem. 279:33593-33600. The purified TNFα
variants were characterized by MALDI-TOF mass spectrometry to verify their identity and
20   integrity.

In situ <u>protease assay: analysis of the protease resistance of TNF-α variants using Western</u>
<u>blot</u>

Aliquots (1 ml) of *E. coli* cell cultures, expressing the various TNF-α variants to be tested,
were centrifuged (5000 g / 5 min / 5°C) and the culture medium was discarded. Cell pellets
25   were resuspended in 1 ml of 20 mM Bis-Tris, pH 6.0. Cell disruption was achieved by
sonication (4 x 15 sec at an amplitude of 11 μm with 20 sec pauses between each sonication
round). The resulting suspensions were centrifuged (20000 g / 30 min / 8° C) and the
supernatants (crude extracts) were transferred to fresh tubes. Crude extracts were then
analysed for protein content using a Bradford protein assay (Biorad). Equivalent amounts of
30   proteins were loaded onto 4-12% NuPAGE gels (Novex). After SDS-PAGE, proteins were
transferred onto nitrocellulose membrane by semi-dry blotting. For immunoreaction,
incubation with rabbit TNFα antiserum (1h; dilution 1:10 000) and with horse radish
peroxidase-conjugated secondary antibody (Dako P448) were carried out in Tris 50mM, NaCl

150mM, EDTA 5mM, Igepal 0.1%, gelatine 0.5%. Detection was with ECL™ reagents (GE Healthcare).

Results

Of the tested TNF variants, three (SEQ ID NOs: 78, 79 and 84) were shown to be protease
resistant in the protease assay (data not shown), evidencing that these TNF variants are not
sensitive to proteolytic cleavage inside the PADRE analogue sequence.

It was concluded that PADRE analogues defined by at least the 13 C-terminal amino acid
residues of each of SEQ ID NOs: 23, 29 and 69 exhibit a desirable increased resistance
towards proteolytic degradation and that proteins modified by including the 13 C-terminal
amino acid residues of SEQ ID NOs: 23, 29 and 69 will exhibit superior yields when produced
recombinantly (because no or only limited fragmentation of the expression product will
occur), provide for a homogenous expression product and possibly provide for increased
immunogenicity because of the increased biological half-life of a protease resistant
immunogen.

EXAMPLE 3

Immunogenicity testing of selected PADRE analogues

The three protease resistant PADRE analogues identified in Example 2 were tested for their
immunogenic properties. In one line of experiments, the peptides were tested as free
peptides in PBMCs (peripheral blood mononuclear cells) from human donors and in two
mouse strains (DR4 and bxd). In the second line of experiments, the PADRE analogues were
tested in the context of the protease resistant TNF variants identified in Example 2.

Test of peptides in human PBMCs

Human PBMCs (peripheral blood mononuclear cells) were stimulated with the peptides with
sequences identical to the 13 amino acid residues of SEQ ID NOs: 23, 29 and 69 (in the
following these 3 13-mers are termed PADRE.Y2.F7.H10, PADRE H2.N7.H10 and
PADRE.I2.H10, respectively). At days 1 and 4 IL-2 is added. At day 7, a portion of he cells
were taken for an IFN-γ ELISPOT assay. The remaining cells were restimulated. At days 8 and
11 IL-2 was added. At day 14, CD4 cells were purified and tested in an IFN-γ ELISPOT assay.

The IFN-γ ELISPOT assay was performed essentially as described in Tangri S *et al.* (2005), J
Immunol **174**, 3187-96.

Test of peptides in mice

Groups of 3 individuals of each of the HLA-DR4 transgenic and bxd mouse strains were immunized with 20 μg (6 μl of 20 mg/ml peptide + 294 μl PBS 1X + 300 μl CFA) or 2 μg (6 μl of 2 mg/ml peptide + 294 μl PBS 1X+ 300 μl CFA) peptide in CFA at the base of the tail. 10

5   to 14 days later the spleens were harvested, CD4 cells purified and tested in an IFN-γ ELISPOT assay (as described in McKinney *et al.* (2004), J Immunol **173**, 1941-50). The peptides tested were identical to those tested in human PBMCs.

Tests of TNFα variants in mice

Groups of three HLA-DR4 transgenic-mice were immunized at the base of the tail with 10 μg

10   of TNF-derived antigen/mouse in 100 μl of complete Freund's adjunvant (CFA). The animals were boosted 14 days after the first immunization using the same dose of antigen in 100 μl of IFA (incomplete Freund's adjuvant). Sera were collected 14 days after each immunization for determination of TNFα specific antibody titers. Animals were then sacrificed for IFN-γ ELISPOT assays.

15   Determination of TNFα specific antibody titers in mice antisera

TNFα specific antibody titers were determined using a direct ELISA. 96-well plates (Maxisorb, Nunc) were coated with recombinant TNFα variant TNF Y87S (5 μg/ml in a carbonate buffer at pH 9.6, 100 μl/well, overnight at 4°C). Plates were then washed three times and incubated for 2hrs at 37°C with 200 μl/well of blocking buffer (phosphate buffer saline containing 1% of

20   bovine serum albumin and 0.05% of Tween 20). Plates were washed three times and pooled sera from mice immunized with human TNFα variants were titrated in six steps, using 1/10-dilution steps (starting dilution is 1/1000) in blocking buffer in a total volume of 100 μl/well. All samples were titrated in duplicates. Serum and control were incubated for 2hr at 37°C. Plates were washed three times and 100 μl of a biotinylated goat anti-mouse IgG (diluted

25   1/10000 in blocking buffer) was transferredd to each well and incubated for 1hr at 37°C. Finally plates were washed three times and incubated for 45 min at room temperature with 100 μl/well of avidin-peroxidase complex (Vectastain Elite Vector PK-6100). Plates were washed again and developed using TMBS. The reaction was stopped after 10-20 min with 100 μl of 4N H_2SO_4 and A_{450} values were determined using an ELISA reader. Antibody titers were

30   defined as the antiserum dilution yielding an A_{450} value of 0.5.

Results

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The results from the test of the peptides in human PBMCs revealed that PADRE.Y2.F7.H10 and PADRE.I2.H10 provided for superior immune responses in the IFN-γ ELISPOT assay at both day 7 and day 14 compared to SEQ ID NO: 3, whereas PADRE.H2.N7.H10 provided for immune responses of the same order of magnitude as did SEQ ID NO: 3.

5   In the tests of PADRE analogue peptides in mice, all 3 tested analogues performed better than PADRE at the 20 µg dose in DR4 mice, whereas only the PADRE.I2.H10 peptide provided for improved immune responses in DR4 transgenic mice at the 2 µg dose as measured by the ELISPOT assay. In the bxd mouse model, all 3 tested PADRE analogues provided for significant immune responses compared to PADRE in the ELISPOT assay at both doses,
10   without however providing for an improvement of the immune response compared to PADRE.

Immunizations with TNFα variants including the PADRE analogues provided for the following results:

The HTL induction in the HLA DR4 transgenic mice as measured by IFN-γ ELISPOT revealed that all TNFα-variants induced HTLs for the PADRE analogue introduced into TNFα and that
15   PADRE.Y2.F7.H10 and PADRE.I2.H10 were superior to PADRE in this respect (where PADRE. H2.N7.H10 provided about the same HTL induction as PADRE). Further, all the variants provided for antibody titers significantly higher than those induced by wild type TNFα - the variants containing PADRE.Y2.F7.H10 and PADRE.I2.H10 were superior compared to PADRE, and again PADRE. H2.N7.H10 provided an immune response comparable to PADRE. In fact,
20   when correlating antibody titers with HTL induction, there was a clear linear correlation.

EXAMPLE 4
Selection and test of 8 further PADRE analogues

Based on the identification of the pan DR binding, protease resistant and immunogenic PADRE analogues PADRE.Y2.F7.H10, PADRE.H2.N7.H10 and PADRE.I2.H10, it was decided to
25   test further PADRE analogues identified as good binders in Example 1. The peptides tested were those having the 13 C-terminal amino acid residues from SEQ ID NOs. 15 (PADRE.F2.N7.H10), 21 (PADRE.Y2.H10), 24 (PADRE.Y2.N7.H10), 26 (PADRE.H2.H10), 47 (PADRE.E2.Y7.H10), 54 (PADRE.N2.N7.H10), 66 (PADRE.Q2.F7.H10), and 72 (PADRE.I2.N7.H10).

30   Three of these sequences were shown to be protease resistant according to the procedure set forth in Example 2, namely PADRE.E2.Y7.H10, PADRE.N2.N7.H10 and PADRE.Q2.N7.H10.

40

Conclusions

The examples described above have demonstrated the existence of a number of PADRE
analogues with improved characteristics in terms of protease resistance and/or
immunogenicity. It is demonstrated that careful modifications in SEQ ID NO: 3 provides for
5   immunogens which are more stable than PADRE, both when being recombinantly expressed
and when being used as *in vivo* immunogens.

It is understood that the examples and embodiments described herein are for illustrative
purposes only and that various modifications or changes in light thereof will be suggested to
persons skilled in the art and are to be included within the spirit and purview of this
10   application and scope of the appended claims. All publications, patents, and patent
applications cited herein are hereby incorporated by reference in their entirety for all
purposes.

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SEQUENCES

SEQ ID NO: 1:	$AX_1FVAAX_2TLX_3AX_4A$	SEQ ID NO: 37:	AAAHFVAAYTLKAEA
SEQ ID NO: 2:	$AX_1FVAAX_2TLHAAA$	SEQ ID NO: 38:	AAAHFVAAFTLKAEA
SEQ ID NO: 3:	AKFVAAWTLKAAA	SEQ ID NO: 39:	AAAHFVAANTLKAEA
SEQ ID NO: 4:	AAAKFVAAWTLKAAA	SEQ ID NO: 40:	AAADFVAAWTLKAAA
SEQ ID NO: 5:	AAAWFVAAWTLKAAA	SEQ ID NO: 41:	AAADFVAAWTLHAAA
SEQ ID NO: 6:	AAAKFVAAWTLHAAA	SEQ ID NO: 42:	AAADFVAAYTLHAAA
SEQ ID NO: 7:	AAAWFVAAWTLHAAA	SEQ ID NO: 43:	AAADFVAAFTLHAAA
SEQ ID NO: 8:	AAAWFVAAYTLHAAA	SEQ ID NO: 44:	AAADFVAANTLHAAA
SEQ ID NO: 9:	AAAWFVAAFTLHAAA	SEQ ID NO: 45:	AAAEFVAAWTLKAAA
SEQ ID NO: 10:	AAAWFVAANTLHAAA	SEQ ID NO: 46:	AAAEFVAAWTLHAAA
SEQ ID NO: 11:	AAAFFVAAWTLKAAA	SEQ ID NO: 47	AAAEFVAAYTLHAAA
SEQ ID NO: 12:	AAAFFVAAWTLHAAA	SEQ ID NO: 48:	AAAEFVAAFTLHAAA
SEQ ID NO: 13:	AAAFFVAAYTLHAAA	SEQ ID NO: 49:	AAAEFVAANTLHAAA
SEQ ID NO: 14:	AAAFFVAAFTLHAAA	SEQ ID NO: 50:	AAANFVAAWTLKAAA
SEQ ID NO: 15:	AAAFFVAANTLHAAA	SEQ ID NO: 51:	AAANFVAAWTLHAAA
SEQ ID NO: 16:	AAAFFVAAWTLKADA	SEQ ID NO: 52:	AAANFVAAYTLHAAA
SEQ ID NO: 17:	AAAFFVAAYTLKADA	SEQ ID NO: 53:	AAANFVAAFTLHAAA
SEQ ID NO: 18:	AAAFFVAAFTLKADA	SEQ ID NO: 54:	AAANFVAANTLHAAA
SEQ ID NO: 19:	AAAFFVAANTLKADA	SEQ ID NO: 55:	AAANFVAAWTLKADA
SEQ ID NO: 20:	AAAYFVAAWTLKAAA	SEQ ID NO: 56:	AAANFVAAYTLKADA
SEQ ID NO: 21:	AAAYFVAAWTLHAAA	SEQ ID NO: 57:	AAANFVAAFTLKADA
SEQ ID NO: 22:	AAAYFVAAYTLHAAA	SEQ ID NO: 58:	AAANFVAANTLKADA
SEQ ID NO: 23:	AAAYFVAAFTLHAAA	SEQ ID NO: 59:	AAANFVAAWTLKAEA
SEQ ID NO: 24:	AAAYFVAANTLHAAA	SEQ ID NO: 60:	AAANFVAAYTLKAEA
SEQ ID NO: 25:	AAAHFVAAWTLKAAA	SEQ ID NO: 61:	AAANFVAAFTLKAEA
SEQ ID NO: 26:	AAAHFVAAWTLHAAA	SEQ ID NO: 62:	AAANFVAANTLKAEA
SEQ ID NO: 27:	AAAHFVAAYTLHAAA	SEQ ID NO: 63:	AAAQFVAAWTLKAAA
SEQ ID NO: 28:	AAAHFVAAFTLHAAA	SEQ ID NO: 64:	AAAQFVAAWTLHAAA
SEQ ID NO: 29:	AAAHFVAANTLHAAA	SEQ ID NO: 65:	AAAQFVAAYTLHAAA
SEQ ID NO: 30:	AAAKFVAAWTLKADA	SEQ ID NO: 66:	AAAQFVAAFTLHAAA
SEQ ID NO: 31:	AAAHFVAAWTLKADA	SEQ ID NO: 67:	AAAQFVAANTLHAAA
SEQ ID NO: 32:	AAAHFVAAYTLKADA	SEQ ID NO: 68:	AAAIFVAAWTLKAAA
SEQ ID NO: 33:	AAAHFVAAFTLKADA	SEQ ID NO: 69:	AAAIFVAAWTLHAAA
SEQ ID NO: 34:	AAAHFVAANTLKADA	SEQ ID NO: 70:	AAAIFVAAYTLHAAA
SEQ ID NO: 35:	AAAKFVAAWTLKAEA	SEQ ID NO: 71:	AAAIFVAAFTLHAAA
SEQ ID NO: 36:	AAAHFVAAWTLKAEA	SEQ ID NO: 72:	AAAIFVAANTLHAAA

SEQ ID NO: 73: VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSE
GLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGA
EAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 74: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO: 75: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAKFVAAWTLKAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 76: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAWFVAANTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 77: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAFFVAANTLKADAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 78: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAYFVAAFTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQV
YFGIIAL

SEQ ID NO: 79: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAHFVAANTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 80: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAHFVAAFTLKAEAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQV
YFGIIAL

SEQ ID NO: 81: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAEFVAAWTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 82: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GANFVAAYTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 83: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAQFVAANTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

SEQ ID NO: 84: MVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVP
SEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSSQTKVNLLSAIKSPCQRETPE
GAIFVAAWTLHAAAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQ
VYFGIIAL

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WHAT IS CLAIMED IS:

1.   An isolated polypeptide comprising an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, wherein the oligopeptide sequence comprises $AX_1FVAAX_2TLX_3AX_4A$ (SEQ ID NO:1), wherein

X$_1$ is selected from the group consisting of W, F, Y, H, D, E, N, Q, I and K;

X$_2$ is selected from the group consisting of F, N, Y and W;

X$_3$ is selected from the group consisting of H and K, and

X$_4$ is selected from the group consisting of A, D and E,

with the proviso that the oligopeptide sequence is not AKFVAAWTLKAAA.

2.   The isolated polypeptide of claim 1, wherein

X$_1$ is selected from the group consisting of Y, H, I, E, N, Q and K;

X$_2$ is selected from the group consisting of F, N, Y and W; and

X$_3$ is H; and

X$_4$ is A.

3.   The isolated polypeptide of claim 1, wherein the oligopeptide has an amino acid sequence identical to the 13 amino acid residue C-terminal fragment in an oligopeptide selected from the group consisting of SEQ ID NOs: 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, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, and 72.

4.   The isolated polypeptide of any one of claims 1-3, wherein the polypeptide, in addition to the oligopeptide, includes a majority of a native polypeptide sequence.

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5.   The isolated polypeptide of claim 4, wherein the native polypeptide sequence is from human TNFα.

6.   The isolated polypeptide of claim 5, wherein the polypeptide has an amino acid sequence selected from any one of SEQ ID NOs76: :, 77, 78, 79, 80, 81, 82, 83, and 84.

5   7.   The isolated polypeptide of any one of claims 1-3, which in addition to the oligopeptide includes a plurality of epitopes from at least one antigen.

8.   The isolated polypeptide of claim 7, wherein the plurality of epitopes is selected from a plurality of CTL epitopes, a plurality of B-cell epitopes, a plurality of T helper lymphocyte epitopes, a plurality of CTL and B-cell epitopes, a plurality of CTL and T helper lymphocyte

10   epitopes, a plurality of B-cell and T helper lymphocyte epitopes, and a plurality of B-cell, CTL and T helper lymphocyte epitopes.

9.   The isolated polypeptide of claim 7 or 8, wherein the epitopes are from one single antigenic protein.

10.   The isolated polypeptide of claim 7 or 8, wherein the epitopes are from at least 2

15   different antigenic proteins.

11.   The isolated polypeptide of claim 10, wherein the at least 2 different antigenic proteins are from the same species.

12.   The isolated polypeptide of any one of claims 1-3 and 6-11, wherein the polypeptide has no more than 100 amino acids.

20   13.   The isolated polypeptide of claim 1 or 4 or 6-11, wherein the polypeptide comprises a second oligopeptide sequence that does not overlap with the oligopeptide sequence that can bind the MHC molecule.

14.   The isolated polypeptide of claim 13, wherein the second oligopeptide sequence comprises a CTL sequence or a B-cell epitope.

25   15.   An isolated polynucleotide that encodes a polypeptide comprising an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, wherein the oligopeptide sequence is as defined in any one of claims 1-14.

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16.   An isolated vector comprising a nucleic acid comprising a promoter operably linked to polynucleotide that encodes a polypeptide comprising an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, wherein the oligopeptide sequence is as defined in any one of claims 1-14.

17.   A cell comprising a nucleic acid comprising a promoter operably linked to polynucleotide that encodes a polypeptide comprising an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, wherein the oligopeptide sequence is as defined in any one of claims 1-14.

18.   The cell according to claim 17, which is an isolated cell.

19.   A composition comprising a polypeptide and an antigen, wherein the polypeptide comprises an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, wherein the oligopeptide sequence comprises is as defined in any one of claims 1-14.

20.   The composition of claim 19, wherein the antigen is a second polypeptide.

21.   The composition of claim 19, wherein the antigen is a non-proteinaceous antigen.

22.   The composition of claim 19, wherein the antigen is a carbohydrate.

23.   A pharmaceutical composition comprising a physiologically acceptable excipient and a polypeptide comprising an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM , wherein the oligopeptide sequence is as defined in any one of claims 1-14.

24.   The pharmaceutical composition of claim 23, further comprising an antigen.

25.   A method of stimulating an immune response, the method comprising administering an effective amount of a polypeptide to an animal, such as a human being, wherein the polypeptide comprises an oligopeptide sequence that can bind an MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than 100 nM, wherein the oligopeptide sequence is as defined in any one of claims 1-14.

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26.   A method of stimulating an immune response, the method comprising administering
an effective amount of a polynucleotide to an animal, such as a human being, wherein the
polynucleotide encodes a polypeptide comprising an oligopeptide sequence that can bind an
MHC class II molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of
5   less than 100 nM, wherein the oligopeptide sequence is as defined in any one of claims 1-14.

27.   A method of stimulating an immune response, the method comprising administering
an effective amount of a non-pathogenic virus or microorganism to an animal, such as a
human being, wherein the microorganism or virus is transformed with a polynucleotide which
encodes a polypeptide comprising an oligopeptide sequence that can bind an MHC class II
10   molecule encoded by at least three different HLA-DR alleles with an IC_{50} value of less than
100 nM, wherein the oligopeptide sequence is as defined in any one of claims 1-14.

SEQ ID NO:	PADRE analog	IC₅₀nM to purified HLA													No. alleles IC₅₀ <1000nM
		DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101	
3		4,0	289	1,4	0,79	32	97	9,5	50	9,1	367	37	447	19	13
4		3,3	209	2,6	2,9	25	49	5,2	28	11	648	9,8	207	5,4	13
5	W4	2,6	161	3,1	3,8	18	82	8,5	35	14	2931	31	566	9,3	12
6	H12	3,9	146	3,4	2,4	17	35	7,6	24	14	656	15	209	22	13
7	W4.H12	4,3	111	2,6	2,0	15	84	8,1	33	32	4240	36	447	28	12
8	W4.Y9.H12	3,9	160	3,8	3,2	24	91	6,8	19	14	3110	6,2	460	25	12
9	W4.F9.H12	5,3	139	3,1	2,5	21	69	10	18	18	21.831	38	115	30	12
10	W4.N9.H12	3,8	174	2,9	5,5	21	43	8,6	33	16	**38**	101	606	22	13
11	F4	5,7	227	2,4	7,3	31	74	12	31	27	2811	32	517	9,9	12
12	F4.H12	3,6	129	2,2	2,9	21	60	7,8	18	14	4183	25	440	13	12
13	F4.Y9.H12	4,4	151	1,7	2,3	19	54	7,4	15	13	5287	4,8	805	11	12
14	F4.F9.H12	5,5	371	2,0	1,1	17	610	82	48	18	218.750	25	129	29	12
15	F4.N9.H12	6,5	285	3,8	4,1	27	82	28	31	42	**64**	70	876	26	13
16	F4.D14	7,2	2838	5,3	2,7	22	91	20	32	65	7053	64	13.928	13	10
17	F4.Y9.D14	3,8	2026	5,0	6,0	33	45	6,7	12	55	856	25	19.927	7,3	11
18	F4.F9.D14	43	49.855	4,5	6,1	8,4	5378	108	113	57	58.333	66	12.459	9,4	9

Fig. 1

SEQ ID NO:	PADRE analog	IC$_{50}$nM to purified HLA													No. alleles IC$_{50}$ <1000nM
		DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101	
19	F4.N9.D14	3,4	140	4,0	4,5	9,5	74	16	18	46	187	103	5522	4,4	12
20	Y4	2,7	108	1,6	1,4	6,6	74	6,1	12	11	917	13	291	5,9	13
21	Y4.H12	3,7	119	2,6	2,6	6,9	55	9,7	18	18	1561	16	279	15	12
22	Y4.Y9.H12	2,5	77	2,3	1,9	7,3	65	5,2	11	12	830	4,0	445	12	13
23	Y4.F9.H12	3,4	126	1,0	0,67	**4,4**	78	5,9	13	17	2555	16	81	18	12
24	Y4.N9.H12	4,5	130	3,3	5,2	13	49	12	21	14	**54**	53	703	16	13
25	H4	4,7	262	3,1	4,8	23	110	12	45	13	1527	20	384	11	12
26	H4.H12	4,2	198	2,5	2,5	12	53	9,3	21	14	1194	12	267	23	12
27	H4.Y9.H12	3,8	214	3,4	5,7	23	94	11	26	16	1321	4,2	526	30	12
28	H4.F9.H12	4,0	228	2,2	3,1	8,6	49	11	24	8,1	1549	13	100	16	12
29	H4.N9.H12	4,1	361	4,5	6,8	18	76	17	37	22	**47**	79	862	33	13
30	D14	5,0	1159	7,3	9,7	17	56	26	27	59	807	19	2389	19	11
31	H4.D14	5,1	1634	8,5	7,1	16	43	24	39	63	875	29	2100	23	11
32	H4.Y9.D14	5,4	1693	11	9,6	25	48	34	31	43	602	11	3798	17	11
33	H4.F9.D14	6,7	2138	15	9,5	19	83	26	30	76	594	37	948	42	12
34	H4.N9.D14	6,1	3116	21	13	36	88	67	60	91	199	172	6394	18	11

Fig. 1 (contd.)

SEQ ID NO:	PADRE analog	IC$_{50}$nM to purified HLA													No. alleles IC$_{50}$ <1000nM
		DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101	
35	E14	5,5	530	6,5	4,7	8,4	59	21	41	36	699	23	710	17	13
36	H4.E14	5,1	1118	6,4	4,7	7,6	54	22	44	53	1103	20	677	18	11
37	H4.Y9.E14	6,9	885	7,5	5,3	11	69	25	35	44	1007	10	721	16	12
38	H4.F9.E14	6,8	855	6,4	8,1	8,1	49	19	46	41	801	31	526	19	13
39	H4.N9.E14	7,1	1843	12	14	36	83	64	59	68	234	255	941	20	12
40	D4	7,0	330	5,1	4,0	30	195	17	57	19	4002	64	1537	20	11
41	D4.H12	4,9	249	5,3	7,1	27	152	24	40	28	4445	89	1533	59	11
42	D4.Y9.H12	4,8	286	4,5	5,3	21	182	14	32	40	4875	19	2480	51	11
43	D4.F9.H12	7,3	746	4,7	3,7	11	223	43	59	44	15.190	97	1047	57	11
44	D4.N9.H12	7,8	731	6,6	8,6	43	196	44	82	57	187	580	4917	80	12
45	E4	2,5	169	3,8	2,8	9,8	81	16	41	45	840	56	332	6,7	13
46	E4.H12	5,1	134	3,1	4,1	27	97	24	44	38	1152	51	350	48	12
47	E4.Y9.H12	3,9	144	2,3	3,2	17	161	13	28	27	1453	6,9	448	52	12
48	E4.F9.H12	6,1	276	2,1	2,6	14	140	19	18	48	2015	50	132	39	12
49	E4.N9.H12	9,0	276	3,5	6,9	44	158	37	87	70	183	137	878	55	13
50	N4	4,4	372	1,8	5,9	18	66	8,5	62	14	1609	12	469	8,7	12

Fig. 1 (contd.)

| SEQ ID NO: | PADRE analog | IC₅₀ nM to purified HLA | | | | | | | | | | | | | No. alleles IC₅₀ <1000nM |
		DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101	
51	N4.H12	4,5	147	1,8	1,5	7,6	53	13	43	11	1167	10	253	20	12
52	N4.Y9.H12	2,7	171	1,8	5,2	19	67	7,5	25	16	1421	4,2	567	20	12
53	N4.F9.H12	4,7	254	1,6	3,9	12	52	12	41	16	2017	16	111	27	12
54	N4.N9.H12	6,1	396	3,4	8,3	48	84	32	72	25	**44**	114	1106	43	12
55	N4.D14	7,6	1664	5,5	9,7	16	52	35	54	41	865	20	2513	24	11
56	N4.Y9.D14	8,5	1529	6,3	15	30	71	22	44	100	819	12	4731	32	11
57	N4.F9.D14	10	2331	4,3	12	13	56	37	55	74	1022	24	1883	20	10
58	N4.N9.D14	8,5	2732	9,7	23	46	98	64	109	128	269	296	6324	29	11
59	N4.E14	1,8	723	2,8	5,8	**3,8**	55	19	58	56	1044	31	221	15	12
60	N4.Y9.E14	6,9	1070	13	6,1	48	68	21	43	60	590	11	718	26	12
61	N4.F9.E14	8,0	1543	5,8	5,0	12	57	14	56	18	1570	29	492	16	11
62	N4.N9.E14	9,3	1743	30	11	46	107	48	64	78	195	359	964	34	12
63	Q4	5,1	271	1,6	2,1	21	58	8,3	39	12	697	17	259	5,2	13
64	Q4.H12	5,0	187	1,5	1,5	13	63	8,3	32	18	1122	13	265	23	12
65	Q4.Y9.H12	4,2	153	2,2	1,9	22	57	5,1	21	17	700	3,6	428	24	13
66	Q4.F9.H12	5,3	325	2,7	4,4	7,7	68	13	31	27	953	18	97	23	13

Fig. 1 (contd.)

SEQ ID NO:	PADRE analog	IC$_{50}$nM to purified HLA													No. alleles IC$_{50}$ ≤1000nM
		DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101	
67	Q4.N9.H12	5,9	319	3,3	6,9	38	76	24	56	44	33	78	525	45	13
68	I4	4,0	393	2,1	1,6	38	75	14	61	25	870	7,3	124	5,6	13
69	I4.H12	6,0	205	2,6	3,7	13	78	12	38	28	891	9,4	84	32	13
70	I4.Y9.H12	5,7	301	2,8	2,6	20	95	9,4	46	19	1950	3,2	346	26	12
71	I4.F9.H12	12	442	1,9	2,7	14	206	36	80	12	6790	9,7	117	21	12
72	I4.N9.H12	7,3	245	2,7	4,5	18	109	27	75	36	65	17	109	37	13

Fig. 1 (contd.)