EPITOPE ANALOGS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/581,001, filed on June 17, 2004, entitled SSX-2 PEPTIDE ANALOGS, and to U.S. Provisional Application No. 60/580,962, filed on June 17, 2004, entitled NY-ESO PEPTIDE ANALOGS; each of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] In certain embodiments, the invention disclosed herein relates to analogs of peptides corresponding to class I MHC-restricted T cell epitopes and methods for their generation. These analogs can contain amino acid substitutions at residues that directly interact with MHC molecules, and can confer improved, modified or useful immunologic properties. In particular, epitope analogs from the tumor-associated antigens SSX-2, NY-ESO-1, PRAME, PSMA, tyrosinase, and melan-A are identified. Additionally classes of analogs, in which the various substitutions comprise the non-standard residues norleucine and/or norvaline, are disclosed.

Description of the Related Art

The Major Histocompatibility Complex and T Cell Target Recognition

[0003] T lymphocytes (T cells) are antigen-specific immune cells that function in response to specific antigen signals. B lymphocytes and the antibodies they produce are also antigen-specific entities. However, unlike B lymphocytes, T cells do not respond to antigens in a free or soluble form. For a T cell to respond to an antigen, it requires the antigen to be bound to a presenting complex known as the major histocompatibility complex (MHC).

[0004] MHC proteins provide the means by which T cells differentiate native or "self" cells from foreign cells. MHC molecules are a category of immune receptors that present potential peptide epitopes to be monitored subsequently by the T cells. There are two types of MHC, class I MHC and class II MHC. CD4+ T cells interact with class II MHC proteins and predominately have a helper phenotype while CD8+ T cells interact with class I MHC proteins and predominately have a cytolytic phenotype, but each of them can also exhibit regulatory, particularly suppressive, function. Both MHC are transmembrane proteins with a majority of their structure on the external surface of the cell. Additionally, both classes of MHC have a peptide binding cleft on their external portions. It is in this cleft that small fragments of proteins, native or foreign, are bound and presented to the extracellular environment.

[0005] Cells called antigen presenting cells (APCs) display antigens to T cells using the MHC. T cells can recognize an antigen, if it is presented on the MHC. This requirement is called MHC restriction. If an antigen is not displayed by a recognizable MHC, the T cell will not recognize and act on the antigen signal. T cells specific for the peptide bound to a recognizable MHC bind to these MHC-peptide complexes and proceed to the next stages of the immune response.

SUMMARY OF THE INVENTION

SSX-2_{41-49} analog embodiments

[0006] Embodiments include analogs of the MHC class I-restricted T cell epitope SSX-2_{41-49}, KASEKIFYV (SEQ ID NO. 1), polypeptides comprising these analogs that can be processed by pAPC to present the epitope analogs, and nucleic acids that express the analogs. The analogs can have similar or improved immunological properties compared to the wild-type epitope.

[0007] One embodiment relates to an isolated SSX-2 peptide having a sequence comprising 1 or more amino acid substitutions of the sequence KASEKIFYV (SEQ ID NO:1), in an amount sufficient to elicit cytokine production from a T cell line generated by immunization against an epitope with the sequence KASEKIFYV (SEQ ID NO:1). In one aspect, the amount sufficient can be not more than 10 uM. In a further aspect, the amount is not more than 3 uM. In a further is not more than 1 uM. In a further aspect, the amount can be not more than 0.3 uM. In one aspect, the substitutions can include a standard amino acid. In a further aspect, the amount can be not more than 0.3 uM. In one aspect, the substitutions can include a standard amino acid, for example Tyr, Val, Leu, Ala, Ile, Met, Trp, Phe, Asp, Asn, or Ser. In a further aspect, the substitutions can include a non-standard amino acid. In one aspect, the non-standard amino acid can be, for example, Nle, Nva, Abu, or a D-stereoisomer of a standard amino acid. In a further aspect, the substitutions can include a modified terminal amino acid. In one aspect, the modified terminal amino acid can be an amidated C-terminal amino acid. In a further aspect at least one of the substitutions can be the addition of an amino acid, wherein the addition is a C-terminal addition. In a further aspect, the peptide further can include the substitution of conserved amino acids at any site, but preferably at the P3, P5, or P7 sites which are not expressly involved in any MHC interactions.

[0008] A further embodiment relates to an isolated peptide of 9 amino acids, P1 to P9, which can include one amino acid at each site. For example, P1 can be K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; P2 can be A, L, V, I, M, D-Ala, Nal-2, Abu, Aib, Nle, or Nva; P3 can be S; P4 can be E, Q, Nle, or Nva; P5 can be K; P6 can be I, L, V, Nle, or Nva; P7 can be F; P8 can be Y, F, Phe(4-F); and PΩ (P-omega) at P9 can be V, I, A, Nva, MeVal, or Abu. In some instances, the sequence is not KASEKIFYV.

[0009] A further embodiment relates to an isolated peptide of 9 amino acids, P1 to P9, which can include one amino acid at each site. For example, P1 can be K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; P2 can be V, L, M, Abu, Nle, or Nva; P3 can

be S; P4 can be E, Q, Nle, or Nva; P5 can be K: P6 can be I, L, V, Nle, or Nva; P7 can be F; P8 can be Y, F, Phe(4-F); and PΩ at P9 can be V, I, A, Nva, MeVal, Abu, or V-NH_2.

[0010] A further embodiment relates to an isolated peptide of 9 amino acids, P1 to P9, which can include one amino acid at each site. For example, P1 can be K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; P2 can be A, L, V, M, Abu, Nle, or Nva; P3 can be S; P4 can be E, Q, Nle, or Nva; P5 can be K; P6 can be I, L, V, Nle, or Nva; P7 can be F; P8 can be Y, F, Phe(4-F); P9 can be V; and PΩ at P10 can be I or L.

[0011] A further embodiment relates to an isolated peptide of 9 amino acids, P1 to P9, which can include one amino acid at each site. For example, P1 can be K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; P2 can be V; P3 can be S; P4 can be E, Q, Nle, or Nva; P5 can be K: P6 can be I, L, V, Nle, or Nva; P7 can be F; P8 can be Y, F, Phe(4-F); P9 can be V; and PΩ at P10 can be I, L, V, or Nle.

[0012] A further embodiment relates to an isolated peptide of 9 amino acids, P1 to P9, which can include one amino acid at each site. For example, P1 can be K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; P2 can be L; P3 can be S; P4 can be E, Q, Nle, or Nva; P5 can be K: P6 can be I, L, V, Nle, or Nva; P7 can be F; P8 can be Y, F, Phe(4-F); P9 can be V; and PΩ at P10 can be I, L, V, Nle or Nva.

[0013] A further embodiment relates to an isolated peptide having the sequence: K{L, V, M, I, D-Ala, D-Val, Nal-2, Aib, Abu, Nle, or Nva}SEKIFYV; or {F, Phg, Y, Phe(4-F), Phe(4-NO_2), O-methyl-Tyr, or β-(3-benzothienyl-Ala}ASEKIFYV; or {Y, F, or W}{V, M, or I}SEKIFYV; or {F or W}LSEKIFYV; or K{A, V, or L}SEKIFYI; or K{L or V}SEKIFYV-NH_2; or FVSEKIFY{I, A, Nva, Abu, or MeVal}; or FVS{Q, Nle, Nva}KIFYV; or FVSEK{L, V, Nle, or Nva}FYV; or FVSEKIF{F, Phe(4-F)}V; or KASEKIFYV{I, L,};or KVSEKIFYV {I, L, V, or Nle}; or KLSEKIFYV {L, V, Nle, or Nva}.

[0014] A further embodiment relates to an isolated peptide having the sequence: K{L, V, M, Abu, Nle, or Nva} SEKIFYV; or {F or Phg}A SEKIFYV; or YVSEKIFYV; or F{L, V, or I}SEKIFYV; or W{L or I}SEKIFYV; or K{V or L}SEKIFYI.; or FVSEKIFY{I or Nva}.

[0015] A further embodiment relates to an isolated peptide having the sequence: K{V or L}SEKIFYV; or {F or Y}ASEKIFYV; or FVSEKIFYI.

[0016] A further embodiment relates to a class I MHC/peptide complex wherein the peptide has the sequence of any of the peptides in the embodiments described above and elsewhere herein. In one aspect, the complex can be cross-reactive with a TCR that recognizes a class I MHC/SSX-2_{41-49} complex. In a further aspect, the complex can be an HLA-A2/SSX-2_{41-49} complex.

[0017] A further embodiment relates an immunogenic composition that can include any of the peptide embodiments described above and elsewhere herein. In one aspect the peptide can have, for example, the sequence: K{L, V, M, Abu, Nle, or Nva} SEKIFYV; or {F or Phg}A SEKIFYV; or YVSEKIFYV; or F{L, V, or I}SEKIFYV; or W{L or I}SEKIFYV; or K{V or

L}SEKIFYI; or FVSEKIFY{I or Nva}, or K{V or L}SEKIFYV; or {F or Y}ASEKIFYV; or FVSEKIFYI.

[0018] Some further embodiments relate to analogs of the MHC class I-restricted T cell epitope NY-ESO-$1_{157-165}$, SLLMWITQC (SEQ ID NO. 1), polypeptides that include these analogs that can be processed by pAPC to present the epitope analogs, and nucleic acids that express the analogs. The analogs can have similar or improved immunological properties compared to the wild-type epitope.

[0019] One embodiment relates to an isolated NY-ESO-$1_{157-165}$ peptide having a sequence comprising 1 or more amino acid substitutions of the sequence SLLMWITQC (SEQ ID NO:1), in an amount sufficient to elicit cytokine production from a T cell line generated by immunization against an epitope with the sequence SLLMWITQC (SEQ ID NO:1). For example, in one aspect, the amount sufficient can be not more than 10 uM. In a further aspect, the amount can be not more than 3 uM. Also, in a further aspect, the amount can be not more than 1 uM. In a further aspect, the amount is not more than 0.3 uM. In one aspect, the substitutions can include a standard amino acid. In a further aspect, the substitutions can include a non-standard amino acid. In one aspect, the non-standard amino acid can be, for example, Tyr, Val, Leu, Ala, Ile, Met, Nle, Nva, Trp, Phe, Asp, Asn, Ser, Abu, and a D-stereoisomer of a standard amino acid. In a further aspect, the substitutions can include a modified terminal amino acid. In one aspect, the modified terminal amino acid can be an amidated C-terminal amino acid. In a further aspect at least one of the substitutions can be the addition of an amino acid, wherein the addition is a C-terminal addition.

[0020] One embodiment relates to an isolated peptide having a sequence in which:

P1 is S, F, K, or W;

P2 is L, I, V, Nle, or Nva;

P3 is L;

P4 is M, L, or N;

P5 is W;

P6 is I, A, L, V, or N;

P7 is T;

P8 is Q, E, D, or T;

PΩ at P9 is C, V, I, L, A, Nva, Nle, V-NH_2, or L-NH_2; and

wherein the sequence is not SLLMWITQ{C, V, I, L, A}, FVLMWITQA, or FILMWITQ{L, I}.

[0021] Another embodiment relates to an isolated peptide having a sequence in which:

P1 is Y;

P2 is L, V, I, Nle, or Nva;

P3 is L;

P4 is M, L, or N;

P5 isW;

P6 is I, A, L, V, or N;

P7 is T;

P8 is Q, E, D, or T;

PΩ at P9 is V, I, L, Nva, Nle, V, V-NH_2, or L-NH_2; and

wherein the sequence is not YVLMWITL or YLLMWIT{I, L}.

[0022] A further embodiment relates to an isolated decamer peptide having a sequence {S,Y}LLMWITQ{C, V}{L, I, Nle}.

[0023] Yet another embodiment relates to an isolated peptide having a sequence SILMWITQ{C, V, L, A}, YLLMWITQ{Nva, Nle}, F{L, V}LMWITQ{V, L, I}, Y{I, Nva, Nle}LMWITQV, YLLLWITQV, or TVLMWITQV.

[0024] A further embodiment relates to an isolated peptide having a sequence {S, F}VLMWITQV, SLMWITQNva, or SNvaLMWITQV

[0025] Still another embodiment relates to an isolated peptide having a sequence SNvaLMWITQV.

[0026] Some embodiments relate to an isolated peptide. The peptide can include or consist essentially of a sequence in which:

P0 is X, XX, or XXX, wherein X specifies any amino acid or no amino acid; and

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is A, L, V, I, M, D-Ala, Nal-2, Abu, Aib, Nle, or Nva; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

PΩ at P9 is V, I, A, Nva, MeVal, Abu, or V-NH_2, or P9 is V, and PΩ at P10 is I, L, V, Nle or Nva; and

PΩ+1 is X, XX, or XXX, wherein X specifies any amino acid or no amino acid; and

wherein the sequence is not KASEKIFYV;

[0027] The isolated peptide can include or consist essentially of the sequence:

K{L, V, M, I, D-Ala, D-Val, Nal-2, Aib, Abu, Nle, or Nva}SEKIFYV; or

{F, Phg, Y, Phe(4-F), Phe(4-NO_2), O-methyl-Tyr, or β-(3-benzothienyl-Ala}ASEKIFYV; or

{Y, F, or W}{V, M, or I}SEKIFYV; or

{F or W}LSEKIFYV; or

K{A, V, or L}SEKIFYI; or

K{L or V}SEKIFYV-NH_2; or

FVSEKIFY{I, A, Nva, Abu, or MeVal}; or

FVS{Q, Nle, Nva}KIFYV; or

FVSEK{L, V, Nle, or Nva}FYV; or

FVSEKIF{F, Phe(4-F)}V; or

KASEKIFYV{I, L,};or

KVSEKIFYV {I, L, V, or Nle}; or

KLSEKIFYV {L, V, Nle, or Nva}.

[0028] The isolated peptide can include or consist essentially of the sequence:

K{L, V, M, Abu, Nle, or Nva} SEKIFYV; or

{F or Phg}A SEKIFYV; or

YVSEKIFYV; or

F{L, V, or I}SEKIFYV; or

W{L or I}SEKIFYV; or

K{V or L}SEKIFYI.; or

FVSEKIFY{I or Nva}.

[0029] Also, the isolated peptide can include or consist essentially of the sequence:

K{V or L}SEKIFYV; or

{F or Y}ASEKIFYV; or

FVSEKIFYI; or

KVSEKIFYV.

[0030] Further, the isolated peptide can include or consist essentially of the sequence KVSEKIFYV.

[0031] The isolated peptide can have affinity for a class I MHC peptide binding cleft. The MHC can be, for example, HLA-A2.

[0032] Some embodiments relate to a class I MHC/peptide complex wherein the peptide can have the sequence of the peptide of claim 1. The class I MHC/peptide complex can be cross-reactive with a TCR that recognizes a class I MHC/SSX-2_{41-49} complex. The class I MHC/peptide complex can be an HLA-A2/SSX-2_{41-49} complex.

[0033] Other embodiments relate to a polypeptide that includes a polypeptide as described above and elsewhere herein, in association with a liberation sequence.

[0034] Still further embodiments relate to immunogenic compositions that include a peptide as described above or elsewhere herein.

[0035] Others relate to nucleic acids encoding or nucleic acid means for expressing a polypeptide as described above or elsewhere herein. Also, some relate to immunogenic compositions that include such nucleic acids or nucleic acid means.

[0036] Some embodiments relate to methods of inducing, maintaining, or amplifying a CTL response. The methods can include intranodal administration of a composition as described above and elsewhere herein.

[0037] Other embodiments relate to methods of entraining a class I MHC-restricted T cell response , which methods can include intranodal administration a composition as described above or elsewhere herein plus an immunopotentiating agent.

[0038] Further embodiments relate to methods of inducing, maintaining, or entraining a CTL response, which methods can include intranodal administration of a composition as described above and elsewhere herein.

[0039] Some embodiments relate to isolated peptides that include 1 to 3 substitutions in the sequence KASEKIFYV having an affinity for a class I MHC binding cleft that is similar to or greater than the affinity of KASEKIFYV for said class I MHC binding cleft. The halftime of dissociation can be similar to or greater than the halftime of dissociation of KASEKIFYV from said class I MHC binding cleft. The isolated peptide can be recognized by T cells with specificity for the peptide KASEKIFYV.

[0040] Still further embodiments relate to isolated peptides that include or consisting essentially of a sequence in which:

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is A, L, V, I, M, D-Ala, Nal-2, Abu, Aib, Nle, or Nva; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

PΩ at P9 is V, I, A, Nva, MeVal, or Abu;

wherein the sequence is not KASEKIFYV;

or

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is V, L, M, Abu, Nle, or Nva; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

PΩ at P9 is V, I, A, Nva, MeVal, Abu, or V-NH_2;

or

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is A, L, V, M, Abu, Nle, or Nva; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

P9 is V; and

PΩ at P10 is I or L;

or

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is V; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

P9 is V; and

PΩ at P10 is I, L, V, or Nle;

or

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is L; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

P9 is V; and

PΩ at P10 is I, L, V, Nle or Nva.

[0041] Some embodiments relate isolated peptides that include or consist essentially of a sequence in which:

P0 is X, XX or XXX, wherein X specifies any amino acid or no amino acid; and

P1 is S, F, K, W or Y; and

P2 is L, I, V, Nle, or Nva; and

P3 is L; and

P4 is M, L, or N; and

P5 is W; and

P6 is I, A, L, V, or N; and

P7 is T; and

P8 is Q, E, D, or T; and

PΩ at P9 is C, V, I, L, A, Nva, Nle, V-NH_2, or L-NH_2; and

PΩ + 1 is X, XX, XXX, wherein X specifies any amino acid or no amino acid; and

wherein the sequence is not SLLMWITQ{C, V, I, L, A}, FVLMWITQA, FILMWITQ{L, I}, YVLMWITL or YLLMWIT{I, L}.

P1 is S, F, K, or W;

P2 is L, I, V, Nle, or Nva;

P3 is L;

P4 is M, L, or N;

P5 is W;

P6 is I, A, L, V, or N;

P7 is T;

P8 is Q, E, D, or T;

PΩ at P9 is C, V, I, L, A, Nva, Nle, V-NH_2, or L-NH_2; and

wherein the sequence is not SLLMWITQ{C, V, I, L, A}, FVLMWITQA, or FILMWITQ{L, I};

or

P1 is Y;

P2 is L, V, I, Nle, or Nva;

P3 is L;

P4 is M, L, or N;

P5 isW;

P6 is I, A, L, V, or N;

P7 is T;

P8 is Q, E, D, or T;

PΩ at P9 is V, I, L, Nva, Nle, V, V-NH_2, or L-NH_2; and

wherein the sequence is not YVLMWITL or YLLMWIT{I, L}.

[0042] A further embodiment relates to a class I MHC/peptide complex wherein the peptide can have the sequence of any of the peptides in the embodiments described above or elsewhere herein. In one aspect, the complex can be cross-reactive with a TCR that recognizes a class I MHC/NY-ESO-$1_{157-165}$ complex. In a further aspect, the complex can be an HLA-A2/NY-ESO-$1_{157-165}$ complex.

[0043] In one aspect of the above embodiments, the peptide can have affinity for a class I MHC peptide binding cleft, such as HLA-A2.

[0044] A further embodiment relates to a polypeptide comprising the peptide sequence of any of the embodiments in association with a liberation sequence.

[0045] A further embodiment relates to an immunogenic composition that includes any of the peptide embodiments. In one aspect the peptide can have a sequence as set forth herein.

[0046] A further embodiment relates to a nucleic acid encoding any of the peptide embodiments, but preferably those which do not have non-standard amino acid substitutions. In a further aspect, the nucleic acid can be encoded in a vector.

[0047] A further embodiment relates to an immunogenic composition that includes the nucleic acid encoding any of the peptide embodiments.

[0048] A further embodiment relates to a method of inducing a CTL response by intranodal administration of any of the compositions or peptides of the embodiments. In a further aspect, the method can allow for maintaining a CTL. In a further aspect, the method can allow for amplifying a class I MHC-restricted T cell response. In a further aspect, the method can allow for entraining a class I MHC-restricted T cell response. In a further aspect, the method also can include an immunopotentiating agent.

[0049] Some embodiments relate to isolated peptides having a sequence comprising 1 to 3 or 4 amino acid substitutions in a native epitope sequence, wherein a concentration of the peptide required to elicit cytokine production from a T cell line generated by immunization against an epitope with the sequence is not more than a particular concentration, for example, 10 uM, 1 uM, 0.3 uM, and the like. The substitutions can include a standard amino acid, a non-standard amino acid, and the like. The non-standard amino acid can be any of those described herein, for example, a D-stereoisomer of a standard amino acid, Nva, or Nle. The substitutions can include a modified terminal amino acid, and the modified terminal amino acid can be an amidated C-terminal amino acid. One of the substitutions can be the addition of an amino acid, for example, the addition can be a C-terminal addition.

[0050] Other embodiments relate to peptides having an amino acid sequence that includes at least one difference from a sequence of a segment of a target-associated antigen, the segment having known or predicted affinity for the peptide binding cleft of a MHC protein, wherein the at least one difference can be a Nle or Nva residue replacing a residue at an MHC-binding motif

anchor position in said segment. The anchor position can be a primary anchor position, for example, P2 or PΩ. The anchor position can be an auxiliary anchor position. The difference can include a Nle or Nva residue replacing a hydrophobic residue in said segment. In some aspects I, L, or V can be a preferred residue in the MHC-binding motif anchor position. In some aspects the peptide can have a length of about 8 to about 14 amino acids or more preferably a length of 9 to 10 amino acids, for example.

[0051] The protein protein can be a human MHC protein, for example, class I MHC protein. The MHC protein can be, for example, a type such as HLA-A2, A3, A24, A30, A66, A68, A69, B7, B8, B15, B27, B35, B37, B38, B39, B40, B48, B51, B52, B53, B 60, B61, B62, B63, B67, B70, B71, B75, B77, C4, Cw1, Cw3, Cw4, Cw6, Cw7, and Cw10. In some aspects, the MHC protein can be HLA-A2 or A24. The MHC can have an anchor residue binding pocket, wherein the pocket is homologous to the B- or F-pocket of HLA-A*0201. The MHC residues responsible for forming binding pockets, and which binding pockets accommodate epitope anchor residues and thus define the binding specificity of the MHC molecule, are well understood in the art. One compilation of such information is found at the FIMM (Functional Immunology) web site at the hypertext transfer protocol (http://) "sdmc.lit.org.sg:8080/fimm/." See also Schönbach C., Koh J.L.Y., Sheng X., Wong L., and V.Brusic. FIMM, a database of functional molecular immunology. Nucleic Acids Research, 2000, Vol. 28, No. 1 222-224; and Schönbach C., Koh JL, Flower DR, Wong L., and Brusic V. FIMM, a database of functional molecular immunology; update 2002. Nucleic Acids Research, 2002, Vol. 30, No. 1 226-229; each of which is hereby incorporated by reference in its entirety. Also, the peptide can have at least one binding characteristic that is substantially the same as, or better than, a corresponding characteristic of said segment for said MHC. For example, the binding characteristic can be elevated compared with that of said segment. Also, the binding characteristic can be affinity or stability of binding for example.

[0052] The peptide can have an immunogenicity that is substantially the same as, or better than, the immunogenicity of the segment. The immunogenicity can be increased. The immunogenicity can evoke an immune response that is cross-reactive to said segment or can evoke a CTL response. The immunogenicity can be assessed, for example, using an MHC-tetramer assay, a cytokine assay, a cytotoxicity assay, by measuring an immune response recognizing the peptide, by measuring an immune response recognizing said segment, using an *in vitro* immunizations system, or any other suitable method. The immunization system can include human cells. The immunogenicity can be assessed using an *in vivo* immunization system, for example, one that includes a transgenic mouse. The peptide can have an at least similar binding characteristic as said segment for said MHC. For example, in some aspects what is considered to be "similar" can be determined based upon the instant disclosure. In some particular aspects "similarity" can be based upon, for example, peptide concentration for half-maximal binding, relative affinity, stability (half time of dissociation) and cross-reactivity/functional avidity. As an example, a peptide can be considered similar if it has

results or characteristics that are within twofold, even threefold, four, five or 10 fold of the value for the native peptide. Also, as an example, for cross-reactivity/functional avidity a similar result can be one where the data are within three and 10-fold of the native peptide. As another example, percentage of binding values can be considered similar when within 2, 3, 4, 5, 6, 7, 10, 15 or 20% of the native peptide. Also, ED50 values can be considered similar in some aspects when within 2- or 3-fold of native sequence. Similar halftime of dissociation can be for example within 2- or 3- fold. As still another example, for cross-reactivity a value that is about 2-fold different from wild-type can be considered similar. These similar values are exemplary only and given in the context of some aspects of some embodiments. Other "similar" values can be determined based upon the other experiments and teachings herein.

[0053] The peptides can be immunologically cross-reactive with the segment. The cross-reactivity can be assessed by immunizing with the segment and assaying recognition of the peptide. The cross-reactivity can be assessed by immunizing with the peptide and assaying recognition of the segment.

[0054] The peptide as described above and elsewhere herein can be modified to include two differences, for example. In some instances each difference independently can include a Nle or Nva residue. In some instances one difference can not include a Nle or Nva residue. Also, the peptide as described above and elsewhere herein can include three or more differences.

[0055] The target-associated antigen can be a tumor-associated antigen. The target-associated antigen can be a pathogen-associated antigen.

[0056] Other embodiments relate to immunogenic composition that include the instant peptides as described above and elsewhere herein. Further embodiments relate to methods of immunization that include administering such compositions to a mammal, for example, administering directly to the lymphatic system.

[0057] Still other embodiments relate to methods of making a T cell epitope analogue. The methods can include providing an amino acid sequence of a segment of a target-associated antigen, the segment can have known or predicted affinity for the peptide binding cleft of a MHC protein; changing at least one amino acid of the sequence corresponding to an anchor position of a MHC binding motif to Nle or Nva; and synthesizing a peptide comprising the changed sequence. The synthesis can be for example, chemical synthesis or any other synthetic method.

[0058] Some embodiments relate to T cell epitopes peptide analogue wherein the analogue differs from a native epitope peptide by replacement of at least one native residue corresponding to an anchor position of a MHC binding motif with a Nle or Nva residue.

[0059] Further embodiments relate to methods to generate and resulting compositions representing peptides that are immune active and carry unnatural amino acids at one or multiple MHC anchor residues.

BRIEF DESCRIPTION OF THE DRAWINGS

[0060] Figures 1A and B summarize the substitutions that have been explored for SSX-2_{41-49} analogs at each individual amino acid position for nonamers and decamers, respectively.

[0061] Figure 2 is a schematic diagram of the methodology of a preferred embodiment for identifying analogs.

[0062] Figure 3 is a table showing the cross-reactivity and functional avidity of SSX-2_{41-49} analogs substituted at a single position.

[0063] Figure 4 is a table showing the cross-reactivity and functional avidity of SSX-2_{41-49} analogs substituted at two positions.

[0064] Figure 5 is a table showing the cross-reactivity and functional avidity of SSX-2_{41-49} analogs substituted at more than two positions.

[0065] Figure 6 is a table showing the cross-reactivity and functional avidity of SSX-2_{41-49} decamer analogs encompassing the nominal 41-49 peptide.

[0066] Figure 7 is a diagram showing the injection schedule of the SSX-2_{41-49} analogs.

[0067] Figure 8 shows the activity of the SSX-2_{41-49} A42V, A42L analogs and wild-type in lysis of tumor cells.

[0068] Figure 9 shows the injections schedule for *in vivo* cytotoxicity studies and *ex vivo* cytotoxicity studies as well as which SSX-2_{41-49} analog peptide was used for the boost.

[0069] Figure 10 is a table showing the *in vivo* specific lysis results for a number of the analogs as compared to a control (wild-type peptide) and EAA (Melan A 26-35).

[0070] Figure 11 is a table showing the *in vivo* specific lysis results for a number of the SSX-2_{41-49} analogs as compared to a control (wild-type peptide) and EAA as well as MHC binding and MHC stability.

[0071] Figure 12 shows the percent specific lysis of tumor cells (624.38 human tumor cells) achieved following immunization with a number of analogs as compared to a wild-type control.

Figure

[0072] Figures 13A-C summarize the substitutions that have been explored at each individual amino acid position for nonamers and decamers, respectively, as well as the results that were obtained.

[0073] Figure 14 is a diagram showing the injection schedule used for analysis and testing of the NY-ESO-1 analogs.

[0074] Figures 15A-C show the specific elimination of target cells as measured by removing the spleens and PBMC from challenged animals and measuring CFSE fluorescence by flow cytometry.

[0075] Figures 16A and B show the *in vivo* cytotoxicity against target cells coated with wild-type peptide after boost with NY-ESO-1 analogs.

[0076] Figures 17A and B show an *ex vivo* analysis of the ability of the analogs to trigger enhanced immunity against the wild-type epitope as assessed by cytokine production.

[0077] Figure 18 illustrates a protocol for validating the antigenicity of the $PSMA_{288-297}$ epitope, as well as the results of the testing.

[0078] Figure 19 is a table showing the cross-reactivity and functional avidity of $PSMA_{288-297}$ analogs substituted at a single position.

[0079] Figure 20 is a table showing the cross-reactivity and functional avidity of $PSMA_{288-297}$ analogs substituted at two positions.

[0080] Figure 21 is a table showing the cross-reactivity and functional avidity of $PSMA_{288-297}$ analogs substituted at more than two positions.

[0081] Figure 22 shows the immunogenicity of various $PSMA_{288-297}$ analogs measured by Elispot.

[0082] Figure 23 shows the results of an assay regarding the amplification of anti-$PSMA_{288-297}$ response by the I297V analog measured by Elispot.

[0083] Figure 24 shows the results of boosting with the I297V analog. The assay showed that the boosting resulted in cytotoxic immunity against a $PSMA^+$ human tumor line.

[0084] Figure 25 illustrates a protocol for validating the antigenicity of the $PRAME_{425-433}$ epitope, as well as the results of the testing.

[0085] Figure 26 is a table showing the cross-reactivity and functional avidity of $PRAME_{425-433}$ analogs substituted at a single position.

[0086] Figure 27 includes two tables (A and B), which show the cross-reactivity and functional avidity of $PRAME_{425-433}$ analogs substituted at two positions.

[0087] Figure 28 is a table showing the cross-reactivity and functional avidity of $PRAME_{425-433}$ analogs substituted at more than two positions.

[0088] Figure 29 shows the immunogenicity of a $PRAME_{425-433}$ analog measured by Elispot.

[0089] Figure 30 shows the results of boosting with the L426Nva L433Nle analog. The assay showed that the boosting resulted in cytotoxic immunity against native epitope coated cells.

[0090] Figure 31 shows a protocol for the *in vivo* evaluation of PRAME analogs, as well as the results of the evaluation.

[0091] Figure 32 shows a protocol for the *ex vivo* stimulation of cytokine production in analog induced, native epitope re-stimulated T cells.

[0092] Figure 33 shows the results of boosting with the L426Nva L433Nle analog. The assay showed that the boosting resulted in cytotoxic immunity against a human tumor cell line.

[0093] Figure 34 depicts a protocol for *in vitro* immunization to $PRAME_{425-433}$.

[0094] Figure 35 shows the tetramer analysis results after *in vitro* immunization with $PRAME_{425-433}$ analogs.

[0095] Figure 36 depicts the structure of the plasmid, pCTLR2, a plasmid that expresses the $PRAME_{425-433}$ epitope.

[0096] Figure 37 shows the assay results for an experiment in which humor tumor cells were lysed by T cells primed with plasmid DNA and boosted with peptides.

[0097] Figure 38 shows the tetramer analysis results after plasmid prime with $Tyr_{369-377}$ and peptide boost with the V377Nva analog.

[0098] Figure 39

[0099] Figure 40 shows a schematic representation of an Tyrosinase analog immunogenicity evaluation protocol.

[0100] Figure 41 shows the immune response results against 624.38 cells contacted with effector cells from HHD primed with plasmid and boosted with $Tyr_{369-377}$ analogs.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0101] Peptides encompassing T cell epitopes are usually poor immunogens or immune modulators due to one of multiple factors: a suboptimal pharmacokinetics profile, limited binding to MHC molecules (reduced K_{on} and increased K_{off}), decreased intrinsic recognition by T cells present in the normal immune repertoire (e.g , through various forms of tolerance). Various strategies have been pursued to improve the immunologic properties of peptides, particularly the screening and use of peptides in which the sequence differs from the natural epitope. Such analogs are known by various names in the art, such as heteroclytic peptides and altered peptide ligands (APL). The generation of such analogs has most often utilized amino acids from the standard set of genetically encoded residues (see for example Valmori, D. et al., *J. Immunol.* 160:1750-1758, 1998). Use of non-standard amino acids has typically been associated with efforts to improve the biochemical stability of the peptide (see, for example, Blanchet, J.-S. et al., *J. Immunol.* 167:5852-5861, 2001).

[0102] Generally, analogs can be categorized into the following two main classes: (1) modification of peptide anchor residues to achieve better HLA binding profiles and higher immune responses, and (2) modification of peptide anchor residues and TCR contact residues to circumvent T cell tolerance for self-antigens.

[0103] Some embodiments relate to analogs that have at least one of the following retained or improved properties, including but not limited to:

1. Cross-reactivity and functional avidity to TCR
2. Affinity for and stability of binding to MHC class I
3. *In vivo* effect on immunity assessed by cytotoxicity
4. *In vivo* effect on immunity assessed by *ex vivo* production of IFN-gamma
5. Increased resistance to proteolysis.

[0104] Some embodiments relate to peptide sequences, including analogs, where the amino acids of the sequence are referred to with a position designator, for example P1, P2, P3, PΩ, etc. In addition, the peptide sequences can be referred to as including a P0 and/or PΩ + 1 designator. In some aspects, P0 can be X, XX, or XXX, where X is any amino acid or no amino acid. Similarly, in some aspects, PΩ + 1 can be X, XX, or XXX, where X is any amino acid or no amino acid. Thus, for example, XXX can mean any combination of any amino acid residues or no amino acid. Thus, these embodiments can encompass polypeptides having up to three additional amino acids (with any combination of amino acid residues) on the N-terminus or C-terminus of the specified sequence. Also, in some aspects, the embodiments can encompass no additional amino acids on the N-terminus or the C-terminus.

[0105] The MHC residues responsible for forming binding pockets, and which binding pockets accommodate epitope anchor residues and thus define the binding specificity of the MHC molecule, are well understood in the art. One compilation of such information is found at the FIMM (Functional Immunology) web site at the hypertext transfer protocol (http://) "sdmc.lit.org.sg:8080/fimm/." See also Schönbach C., Koh J.L.Y., Sheng X., Wong L., and V.Brusic. FIMM, a database of functional molecular immunology. Nucleic Acids Research, 2000, Vol. 28, No. 1 222-224; and Schönbach C., Koh JL, Flower DR, Wong L., and Brusic V. FIMM, a database of functional molecular immunology; update 2002. Nucleic Acids Research, 2002, Vol. 30, No. 1 226-229; each of which is hereby incorporated by reference in its entirety.

[0106] The phrase "liberation sequence" refers to a peptide comprising or encoding an epitope or an analog, which is embedded in a larger sequence that provides a context allowing the epitope or analog to be liberated by immunoproteasomal processing, directly or in combination with N-terminal trimming or other physiologic processes. In some aspects, the analog or epitope can be designed or engineered.

[0107] Other embodiments relate to epitope arrays and other polypeptides that include the epitope analog sequences that can be processed to liberate the analog. Further embodiments relate to nucleic acids, particularly DNA plasmids, encoding such polypeptides, or simply an analog, and their expression therefrom. The analogs, the polypeptides comprising them, and the encoding nucleic acids can all be components of immunogenic compositions, particularly compositions suitable for intralymphatic delivery, all of which relate to further embodiments.

[0108] Peptide analogs with improved immunologic properties can be designed by modifying the anchor residues involved in the interaction with MHC molecules, so as to increase the binding and stabilize the formation of MHC-peptide complexes. Such modifications can be guided by knowledge of the binding motif or preferred anchor residues of the restricting MHC molecule. There further exist various rules, indexes and algorithms that can be used to predict the properties of analogs bearing various substitutions with the limitation that the substitution is selected from the standard set of genetically encodable amino acids.

[0109] However, there are no databases or algorithms to predict the outcome of replacing anchor residues with non-standard amino acids and their usefulness is previously not well explored. It is herein disclosed that the non-standard amino acids norleucine (Nle) and norvaline (Nva) can be advantageously substituted into the anchor residue positions of MHC-binding peptides. It is preferred that they be placed in a position favorably occupied by a hydrophobic or a large amino acid, especially I, L, or V.

[0110] MHC-binding motifs are generally defined in terms of preferred residue side chains at nominal positions within a span of 8 to 10 amino acids (see for example Rammensee et al., "MHC Ligands and Peptide Motifs," (Molecular Biology Intelligence Unit), Springer-Verlag, Germany, 1997 Landes Bioscience, Austin, Texas; and Parker, et al., "Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains," J. Immunol. 152:163-175. Website algorithms are also available which can be used to predict MHC binding. See for example, the world wide web page of Hans-Georg Rammensee, Jutta Bachmann, Niels Emmerich, Stefan Stevanovic: SYFPEITHI: An Internet Database for MHC Ligands and Peptide Motifs (hypertext transfer protocol access via: syfpeithi.bmi-heidelberg.com/scripts/MHCServer.dll/home.htm) and another is "bimas.dcrt.nih.gov/molbio/hla_bind." For class I-restricted epitopes the C-terminal position, PΩ, is typically a primary anchor. The 2nd position, P2, is also often a primary anchor or, alternatively, P3 and/or P5 can serve this role. Positions P2 through P7 have all been recognized as secondary or auxiliary anchor positions for one or another MHC (see Rammensee et al., and see Table 6 from U.S. Patent Application Publication No. 2003-0215425 (U.S. Patent Application No. 10/026,066, filed on December 7, 2001, entitled EPITOPE SYNCHRONIZATION IN ANTIGEN PRESENTING CELLS; which is incorporated herein by reference in its entirety for all of its disclosure). For class II-restricted epitopes P1, P4, P6, P7, and P9 have been recognized as anchor positions. The foregoing is intended as a general guide and should be considered exemplary and not exhaustive or limiting. Many analyses and compilations of binding motifs, anchor residues, and the like are available in the scientific and patent literature and over the internet. Their conventions and results further provide those of skill in the art useful guide to the design of epitope analogs, when coupled with the teaching herein.

[0111] The length of the peptide actually bound to the presenting MHC molecule can be longer than the nominal motif sequence. The ends of the binding cleft for class II MHC molecules are open so that the bound peptide can be extended at either end of the core motif. In contrast the binding cleft is closed at both ends in class I MHC molecules so that the ends of the bound peptide must generally correspond to the motif, however significant variation in length can be accommodated through bulging and folding of the central region of the bound peptide, so that peptides of up to at least about 14 amino acids in length can be presented (see for example Probst-Kepper, M. et al., *J. Immunol.* 173:5610-5616, 2004).

[0112] Epitope analogs can have improved K_{on} and K_{off} related to the interaction with class I MHC molecules, as well as preserved or increased interaction with T cell receptors recognizing the original epitope, modified or improved *in vivo* or *ex vivo* activity reflected in enhanced expansion of specific T cell populations, improved cytokine production by specific T cells, or *in vivo* or *in vitro* cytotoxicity against targets carrying natural epitopes, mediated by T cells that reacted with the peptide. In addition, such analogs may interact in a more optimal fashion with multiple distinct MHC class I molecules.

[0113] Such peptide analogs with improved immune properties may encompass one or multiple substitutions, including one or multiple non-standard amino acids. Among non-standard amino acids, substitutions for primary anchor residues consisting of norvaline or norleucine are preferred since, as exemplified below, they can not only improve on the interaction with MHC class I, but can also preserve cross-reactivity with TCR specific for the native epitope and show improved *in vivo* immune profile. More specifically, mutating the P2 amino acid residue from A, L or V to norvaline or norleucine improved immune properties and is thus preferred. In addition, modifying the C terminal residue to norvaline or preferably norleucine, improved immune features of the analogs. In addition, analogs that encompass multiple substitutions at primary and/or secondary anchor residues including norvaline and/or norleucine at P2 or PΩ, can be associated with improved immune properties.

[0114] Certain uses of norvaline (Nva) and norleucine (Nle) are mentioned in U.S. Patent No. 6,685,947, PCT Publication Nos. WO 03/076585 A2 and WO 01/62776 A1 and U.S. Patent Publication No. 20040253218A1. None of these references teaches the general usefulness of Nva or Nle substituted at an anchor position of a MHC-biding peptide to improve an immunological property. The '218 publication teaches that the substituted residues should be incorporated at TCR-interacting positions and not at MHC-interacting positions:

> [0111] In still another embodiment of the invention, the peptide is an analog of a peptide derived from an NS-specific antigen that is immunogenic but not encephalitogenic. The most suitable peptides for this purpose are those in which an encephalitogenic self-peptide is modified at the T-cell receptor (TCR) binding site and not at the MHC binding site(s), so that the immune response is activated but not anergized (Karin et al, 1998; Vergelli et al, 1996).

[0115] HLA-A2.1-restricted peptides incorporating Nle disclosed in the '776 publication are derived from CEA, p53, and MAGE-3. In the CEA peptide I(Nle)GVLVGV and the p53 peptide S(Nle)PPPGTRV, Nle is present at the P2 position. No teaching about the general usefulness of norleucine is provided and no disclosure is provided indicating how or if these substitutions altered the properties of the analogs as compared to the native sequence.

[0116] Some of the instant embodiments relate to epitope analogs that incorporate Nva and/or Nle at a position promoting binding to MHC. Some embodiments specifically exclude the use

Nle and/or Nva in HLA-A2.1-restricted epitopes, HLA-A2.1 epitopes from CEA, p53, and/or MAGE-3, or other peptides derived from MAGE-3, CEA, and/or p53. In some embodiments, one or more of the specific sequences as disclosed in the above referenced patent references are specifically excluded. Other exemplary embodiments include the use of Nle and/or Nva at P3, P5, and/or PΩ anchor positions, at an auxiliary anchor position, to make an analog of a non-A2- or non-A2.1-HLA restricted epitope, in an anchor position of a peptide that is not derived from an oncogene or oncofetal protein, and in an anchor position of a peptide derived from a CT antigen.

[0117] In general, such analogs may be useful for immunotherapy and/or prophylaxis of various diseases such as infectious, cancerous or inflammatory, as single agents or in combination therapies, due to their optimized interaction with MHC molecules and T cell receptors, key to onset and regulation of immune responses.

Analog production

[0118] The analogs may be produced using any method known to one of skill in the art, including manufacturing the peptides using a method of peptide synthesis or expressing nucleic acids that code for the desired peptide analogs. Thus, when the analogs include one or more non-standard amino acids, it is more likely that they will be produced by a method of peptide production. When the analogs include only one or more substitutions with standard amino acids, they may be expressed from an expression vector using any method known to one of skill in the art. Alternatively, the peptides may be expressed using a method of gene therapy.

Analog testing

[0119] The usefulness and/or the activity of the analogs was identified. In this way useful and/or improved analogs can be identified. To be useful, an analog may not necessarily be found to be improved in the identified assays. Thus, a useful peptide may contain other properties such as being useful in a tolerized patient or resistant to proteolysis. To be improved, a peptide can be found to have a clear improvement in binding to the TCR, binding to the MHC molecule, and an improved immune response or any other biological activity. To be useful, the peptide may be found not to be improved when using a murine test system, but because of the differences in the human immune system, may be improved when tested in a human. Alternatively, the usefulness may stem from a potential to break tolerance in a tolerized human. Alternatively, the usefulness may stem from the ability to use the peptide as a base for further substitutions to identify improved analogs.

[0120] In order to evaluate usefulness, improved properties and to compare the analogs in any way to the wild-type, one or more of the following assays were conducted: peptide binding affinity for HLA-A*0201; peptide-HLA-A*0201 complex stability assay; cross-reactivity assay (recognition of peptide analogs by wild-type peptide specific CTL or recognition of wild-type peptide by CTL generated using peptide analogs); immunogenicity assays, such as an IFN-γ secretion

assay, a cytotoxicity assay, and/or an Elispot assay; antigenicity assays such as an *in vitro* tumor cell lysis assay, an *ex vivo* tumor cell lysis, and an *in vivo* tumor cell lysis; and proteolysis assays to identify increased resistance to proteolysis. Details of exemplary assays are presented in the Examples.

Uses of the Analogs

[0121] Useful methods for using the disclosed analogs in inducing, entraining, maintaining, modulating and amplifying class I MHC-restricted T cell responses, and particularly effector and memory CTL responses to antigen, are described in U.S. Patent application Nos. 09/380,534 and 09/776,232 both entitled A METHOD OF INDUCING A CTL RESPONSE; U.S. Provisional Application No. 60/479,393, filed on June 17, 2003, entitled METHODS TO CONTROL MHC CLASS I-RESTRICTED IMMUNE RESPONSE; and U.S. Patent Application No. 10/871,707 (Pub. No. 2005 0079152) and Provisional U.S. Patent Application No. 60/640,402 filed on December 29, 2004, both entitled METHODS TO ELICIT, ENHANCE AND SUSTAIN IMMUNE RESPONSES AGAINST MHC CLASS I-RESTRICTED EPITOPES, FOR PROPHYLACTIC OR THERAPEUTIC PURPOSE. The analogs can also be used in research to obtain further optimized analogs. Numerous housekeeping epitopes are provided in U.S. Application Nos. 10/117,937, filed on April 4, 2002 (Pub. No. 20030220239 A1), and 10/657,022 (2004-0180354), and in PCT Application No. PCT/US2003/027706 (Pub. No. WO04022709A2), filed on September 5, 2003; and U.S. Provisional Application Nos. 60/282,211, filed on April 6, 2001; 60/337,017, filed on November 7, 2001; 60/363,210 filed on March 7, 2002; and 60/409,123, filed on September 5, 2002; each of which applications is entitled EPITOPE SEQUENCES. The analogs can further be used in any of the various modes described in those applications. Epitope clusters, which may comprise or include the instant analogs, are disclosed and more fully defined in U.S. Patent Application No. 09/561,571, filed on April 28, 2000, entitled EPITOPE CLUSTERS. Methodology for using and delivering the instant analogs is described in U.S. Patent applications 09/380,534 and 09/776,232 (Pub. No. 20020007173 A1), and in PCT Application No. PCTUS98/14289 (Pub. No. WO9902183A2) each entitled A METHOD OF INDUCING A CTL RESPONSE. Beneficial epitope selection principles for such immunotherapeutics are disclosed in U.S. Patent Application Nos. 09/560,465, filed on April 28, 2000, 10/026,066 (Pub. No. 20030215425 A1), filed on December 7, 2001, and 10/005,905 filed on November 7, 2001, all entitled EPITOPE SYNCHRONIZATION IN ANTIGEN PRESENTING CELLS; 09/561,074 entitled METHOD OF EPITOPE DISCOVERY; 09/561,571, filed April 28, 2000, entitled EPITOPE CLUSTERS; 10/094,699 (Pub. No. 20030046714 A1), filed March 7, 2002, entitled ANTI-NEOVASCULATURE PREPARATIONS FOR CANCER; Application Nos. 10/117,937 (Pub. No. 20030220239 A1) and PCTUS02/11101 (Pub. No. WO02081646A2), both filed on April 4, 2002, and both entitled EPITOPE SEQUENCES; and Application Nos. 10/657,022 and PCT Application No. PCT/US2003/027706 (Pub. No.

WO04022709A2), both filed on September 5, 2003, and both entitled EPITOPE SEQUENCES. Aspects of the overall design of vaccine plasmids are disclosed in U.S. Patent Application Nos. 09/561,572, filed on April 28, 2000, entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS and 10/292,413 (Pub. No.20030228634 A1), filed on November 7, 2002, entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS AND METHODS FOR THEIR DESIGN; 10/225,568 (Pub No. 2003-0138808) filed on August 20, 2002, PCT Application No. PCT/US2003/026231 (Pub. No. WO 2004/018666) filed on August 19, 2003, both entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS; and U.S. Patent No. 6,709,844, entitled AVOIDANCE OF UNDESIRABLE REPLICATION INTERMEDIATES IN PLASMID PROPAGATION. Specific antigenic combinations of particular benefit in directing an immune response against particular cancers are disclosed in Provisional U.S. patent Application No. 60/479,554 filed on June 17, 2003 and U.S. Patent Application No. 10/871,708 filed on June 17, 2004 and PCT Patent Application No. PCT/US2004/019571 (Pub. No. WO 2004/112825), all entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN VACCINES FOR VARIOUS TYPES OF CANCERS. Antigens associated with tumor neovasculature (*e.g.*, PSMA, VEGFR2, Tie-2) are also useful in connection with cancerous diseases, as is disclosed in U.S. Patent Application No. 10/094,699 (Pub. No. 20030046714 A1), filed March 7, 2002, entitled ANTI-NEOVASCULATURE PREPARATIONS FOR CANCER. Methods to trigger maintain and manipulate immune responses by targeted administration of biological response modifiers are disclosed U.S. Provisional Application No. 60/640,727, filed on December 29, 2004. Methods to bypass CD4+ cells in the induction of an immune response are disclosed in U.S. Provisional Application No. 60/640,821, filed on December 29, 2004. Exemplary diseases, organisms and antigens and epitopes associated with target organisms, cells and diseases are described in U.S. Application No. 09/776,232 (Pub. No. 20020007173), filed February 2, 2001 and entitled METHOD OF INDUCING A CTL RESPONSE. Exemplary methodology is found in U.S. Provisional Application No. 60/580,969, filed on June 17, 2004, and U.S. Patent Application No. __/_____ (Attorney Docket: MANNK.050A), filed on June 17, 2005, both entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOTISTICS FOR VARIOUS TYPES OF CANCERS. Methodolgoy and compositions are also disclosed in U.S. Provisional Application No. 60/640,598, filed on December 29, 2004, entitled COMBINATIONS OF TUMOR-ASSOCAITED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCER. The integration of diagnostic techniques to assess and monitor immune responsiveness with methods of immunization including utilizing the instant analogs is discussed more fully in Provisional U.S. Patent Application No. 60/580,964 filed on June 17, 2004 and U.S. Patent Application No. __/_____ (Atty. Docket No. MANNK.040A), filed on June 17, 2005, both entitled IMPROVED EFFICACY OF ACTIVE IMMUNOTHERAPY BY INTEGRATING DIAGNOSTIC WITH THERAPEUTIC METHODS.

The immunogenic polypeptide encoding vectors are disclosed in U.S. Patent application No. 10/292,413 (Pub. No. 20030228634 A1) filed on November 7, 2002 entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS AND METHODS FOR THEIR DESIGN, and in U.S. Provisional Application No. __/___,___, filed on June 17, 2005, entitled (Attorney Docket No. MANNK.053PR), entitled METHODS AND COMPOSITIONS TO ELICIT MULTIVALENT IMMUNE RESPONSES AGAINST DOMINANT AND SUBDOMINANT EPITOPES, EXPRESSED ON CANCER CELLS AND TUMOR STROMA. Additional useful disclosure, includes methods and compositions of matter is found in U.S. Provisional Application No. __/___,___, filed on June 17, 2005 (Attorney Docket No. MANNK.054PR), entitled MULTIVALENT ENTRAIN-AND-AMPLIFY IMMUNOTHERAPEUTICS FOR CARCINOMA. Further methodology, compositions, peptides, and peptide analogs are disclosed in U.S. Provisional Application Nos. 60/581,001 and 60/580,962, both filed on June 17, 2004, and respectively entitled "SSX-2 PEPTIDE ANALOGS" and "NY-ESO PEPTIDE ANALOGS." All of these applications mentioned in this paragraph are hereby incorporated by reference in their entirety for all that they teach. Additional analogs, peptides and methods are disclosed in U.S. Patent Application No. __/___,___ (Attorney Docket: MANNK.038A) entitled SSX-2 PEPTIDE ANALOGS; U.S. Patent Application No. __/___,___ (Attorney Docket: MANNK.039A), entitled NY-ESO-1 PEPTIDE ANALOGS; and U.S. Patent Application No. __/___,___(Attorney Docket: MANNK.051A), entitled EPITOPE ANALOGS; all filed on June 17, 2005. As an example, without being limited thereto the references are incorporated by reference for what they teach about class I MHC-restricted epitopes, analogs, the design of analogs, uses of epitopes and analogs, methods of using and making epitopes, and the design and use of nucleic acid vectors for their expression.

Antigens

[0122] There are many antigens, epitopes of which can be recognized by T cells in an MHC-restricted manner, for which manipulation of an immune response directed against them has therapeutic or prophylactic potential. The principles for making analogs of MHC-binding peptides described herein are generally applicable to any of these antigens and their epitopes. A particular focus of the present disclosure is epitopes from the tumor-associated antigens (TuAA) SSX-2, NY-ESO-1, PRAME, PSMA, tyrosinase, and Melan-A.

[0123] SSX-2, also know as Hom-Mel-40, is a member of a family of highly conserved cancer-testis antigens (Gure, A.O. et al. Int. J. Cancer 72:965-971, 1997, which is hereby incorporated by reference in its entirety). Its identification as the TuAA antigen is taught in U.S. Patent 6,025,191 entitled ISOLATED NUCLEIC ACID MOLECULES THAT ENCODE A MELANOMA SPECIFIC ANTIGEN AND USES THEREOF, which is hereby incorporated by reference in its entirety. Cancer-testis antigens are found in a variety of tumors, but are generally

absent from normal adult tissues except testis. SSX-2 is expressed in many different types of tumors, including synovial sarcomas, melanoma, head and neck cancers, breast, colon and ovarian cancers. In addition to its widespread expression in a variety of cancers, it is also immunogenic in patients with late stage disease. Further, there is evidence of spontaneous humoral and cellular immune responses towards this antigen in metastatic tumor patients (Ayyoub M, et al., Cancer Res. 63(17): 5601-6, 2003; Ayyoub M, et al. J Immunol. 168(4): 1717-22, 2002), which is incorporated herein by reference in its entirety. Two HLA-A2 restricted T cell epitopes have been identified recently using reverse T-cell immunology, namely SSX-2_{41-49} (Ayyoub M, et al. J Immunol. 168(4): 1717-22, 2002; U.S. Patent No.6548064, entitled ISOLATED PEPTIDES CONSISTING OF AMINO ACID SEQUENCES FOUND IN SSX OR NY-ESO-1 MOLECULES, THAT BIND TO HLA MOLECULE; U.S. Patent Application No. 10/117,937, entitled EPITOPE SEQUENCES) and SSX-$2_{103-111}$ (Wagner C, et al. Cancer Immunity 3:18, 2003), each of which is incorporated herein by reference in its entirety. The C-termini of both epitopes can be efficiently generated by *in vitro* proteasome digestion. Isolated HLA-A*0201/SSX-2_{41-49} multimer$^+$ CD8$^+$ T cells from tumor-infiltrated lymph nodes of SSX-2 positive patients exhibited high functional avidity and can effectively recognize SSX-2 positive tumors; however, the spontaneously occurring immunological responses were not sufficient for stopping tumor growth, possibly because these immune response did not develop until fairly late in the disease progression, and the activated T cells were not numerous enough. U.S. Patent No. 6,548,064 (which is incorporated herein by reference in its entirety) further describes substituting a T or A residue at both the P2 and PΩ position of an SSX-2 epitope.

[0124] NY-ESO-1 is a cancer-testis antigen found in a wide variety of tumors and is also known as CTAG-1 (Cancer-Testis Antigen-1) and CAG-3 (Cancer Antigen-3). NY-ESO-1 as a tumor-associated antigen (TuAA) is disclosed in U.S. Patent 5,804,381 entitled ISOLATED NUCLEIC ACID MOLECULE ENCODING AN ESOPHAGEAL CANCER ASSOCIATED ANTIGEN, THE ANTIGEN ITSELF, AND USES THEREOF that is hereby incorporated by reference in its entirety. A paralogous locus encoding antigens with extensive sequence identity, LAGE-1a/s and LAGE-1b/L, have been disclosed in publicly available assemblies of the human genome, and have been concluded to arise through alternate splicing. Additionally, CT-2 (or CTAG-2, Cancer-Testis Antigen-2) appears to be either an allele, a mutant, or a sequencing discrepancy of LAGE-1b/L. Due to the extensive sequence identity, many epitopes from NY-ESO-1 can also induce immunity to tumors expressing these other antigens. The proteins are virtually identical through amino acid 70. From 71-134 the longest run of identities between NY-ESO-1 and LAGE is 6 residues, but potentially cross-reactive sequences are present. And from 135-180 NY-ESO and LAGE-1a/s are identical except for a single residue, but LAGE-1b/L is unrelated due to the alternate splice. The CAMEL and LAGE-2 antigens appear to derive from the LAGE-1 mRNA, but from alternate reading frames, thus giving rise to unrelated protein sequences. More recently, GenBank

Accession AF277315.5 (which is incorporated herein by reference in its entirety), Homo sapiens chromosome X clone RP5-865E18, RP5-1087L19, complete sequence, reports three independent loci in this region that are labeled as LAGE1 (corresponding to CTAG-2 in the genome assemblies), plus LAGE2-A and LAGE2-B (both corresponding to CTAG-1 in the genome assemblies).

[0125] NY-ESO-$1_{157-165}$ is identified as an HLA-A2 restricted epitope in U.S. Patent No. 6,274,145 entitled ISOLATED NUCLEIC ACID MOLECULE ENCODING CANCER ASSOCIATED ANTIGEN, THE ANTIGEN ITSELF, AND USES THEREOF, and U.S. Patent Application No. 10/117,937 (Pub. No. 20030220239) entitled EPITOPE SEQUENCES reports that this C-terminus is generated by the housekeeping proteasome in an in vitro assay. Analogs substituting A,V,L,I,P,F,M,W, or G at PΩ, alone or in combination with A at another position, are disclosed in U.S. Patent Nos. 6,417,165 and 6,605,711, both entitled NY-ESO-1-PEPTIDE DERIVATIVES, AND USES THEREOF. The references described in this paragraph are incorporated herein by reference in their entirety.

[0126] PRAME, also know as MAPE, DAGE, and OIP4, was originally observed as a melanoma antigen. Subsequently, it has been recognized as a CT antigen, but unlike many CT antigens (e.g., MAGE, GAGE, and BAGE) it is expressed in acute myeloid leukemias. PRAME is a member of the MAPE family which consists largely of hypothetical proteins with which it shares limited sequence similarity. The usefulness of PRAME as a TuAA is taught in U.S. Patent No. 5,830,753 entitled ISOLATED NUCLEIC ACID MOLECULES CODING FOR TUMOR REJECTION ANTIGEN PRECURSOR DAGE AND USES THEREOF, which is hereby incorporated by reference in its entirety. U.S. Patent Application No. 10/181,499, entitled METHODS FOR SELECTING AND PRODUCING T CELL PEPTIDE EPITOPES AND VACCINES INCORPORATING SAID SELECTED EPITOPES (which is incorporated herein by reference in its entirety) identifies a variety of potential epitopes, including $PRAME_{425-433}$, using in vitro digestion with immunoproteasome.

[0127] PSMA (prostate-specific membranes antigen), a TuAA described in U.S. Patent 5,538,866 entitled "PROSTATE-SPECIFIC MEMBRANES ANTIGEN" which is hereby incorporated by reference in its entirety, is expressed by normal prostate epithelium and, at a higher level, in prostatic cancer. It has also been found in the neovasculature of non-prostatic tumors. PSMA can thus form the basis for vaccines directed to both prostate cancer and to the neovasculature of other tumors. This later concept is more fully described in U.S. Patent Publication No. 20030046714; PCT Publication No. WO 02/069907; and a provisional U.S. Patent application No. 60/274,063 entitled ANTI-NEOVASCULAR VACCINES FOR CANCER, filed March 7, 2001, and U.S. Application No. 10/094,699, filed on March 7, 2002, entitled "ANTI-NEOVASCULAR PREPARATIONS FOR CANCER," each of which are hereby incorporated by reference in its entirety. The teachings and embodiments disclosed in said publications and applications are contemplated as supporting principals and embodiments related to and useful in connection with the

present invention. Briefly, as tumors grow they recruit ingrowth of new blood vessels. This is understood to be necessary to sustain growth as the centers of unvascularized tumors are generally necrotic and angiogenesis inhibitors have been reported to cause tumor regression. Such new blood vessels, or neovasculature, express antigens not found in established vessels, and thus can be specifically targeted. By inducing CTL against neovascular antigens the vessels can be disrupted, interrupting the flow of nutrients to (and removal of wastes from) tumors, leading to regression.

[0128] Alternate splicing of the PSMA mRNA also leads to a protein with an apparent start at Met_{58}, thereby deleting the putative membrane anchor region of PSMA as described in U.S. Patent 5,935,818 entitled "ISOLATED NUCLEIC ACID MOLECULE ENCODING ALTERNATIVELY SPLICED PROSTATE-SPECIFIC MEMBRANES ANTIGEN AND USES THEREOF" which is hereby incorporated by reference in its entirety. A protein termed PSMA-like protein, Genbank accession number AF261715, which is hereby incorporated by reference in its entirety, is nearly identical to amino acids 309-750 of PSMA and has a different expression profile. Thus the more preferred epitopes are those with an N-terminus located from amino acid 58 to 308. $PSMA_{288-297}$ was identified as possessing an HLA-A2 binding motif in WO 01/62776, entitled HLA BINDING PEPTIDES AND THEIR USES, which is hereby incorporated by reference in its entirety. Its production *in vitro* by digestion with housekeeping proteasome and actual binding to HLA-A2 was disclosed in U.S. Patent Application Publication No. 2003-0220239 entitled EPITOPE SEQUENCES.

[0129] Tyrosinase is a melanin biosynthetic enzyme that is considered one of the most specific markers of melanocytic differentiation. Tyrosinase is expressed in few cell types, primarily in melanocytes, and high levels are often found in melanomas. The usefulness of tyrosinase as a TuAA is taught in U.S. Patent 5,747,271 entitled "METHOD FOR IDENTIFYING INDIVIDUALS SUFFERING FROM A CELLULAR ABNORMALITY SOME OF WHOSE ABNORMAL CELLS PRESENT COMPLEXES OF HLA-A2/TYROSINASE DERIVED PEPTIDES, AND METHODS FOR TREATING SAID INDIVIDUALS" which is hereby incorporated by reference in its entirety.

[0130] Melan-A, also called MART-1 (Melanoma Antigen Recognized by T cells), is another melanin biosynthetic protein expressed at high levels in melanomas. The usefulness of Melan-A/MART-1 as a TuAA is taught in U.S. Patent Nos. 5,874,560 and 5,994,523 both entitiled MELANOMA ANTIGENS AND THEIR USE IN DIAGNOSTIC AND THERAPEUTIC METHODS, as well as U.S. Patent No. 5,620,886, entitled ISOLATED NUCLEIC ACID SEQUENCE CODING FOR A TUMOR REJECTION ANTIGEN PRECURSOR PROCESSED TO AT LEAST ONE TUMOR REJECTION ANTIGEN PRESENTED BY HLA-A2, all of which are hereby incorporated by reference in their entirety. The immunodominant HLA-A2 restricted epitope from this TuAA is Melan-A_{26-35}. It has been shown to be produced by the housekeeping proteasome (Morel, S. et al. *Immunity* 12:107-117, 2000), which is hereby incorporated by reference in its entirety. Various analogs incorporating standard amino acids, including an improved analog

substituting L at P2, are disclosed in U.S. Patent No. 6,025,470, entitled ISOLATED NONA- AND DECAPEPTIDES WHICH BIND TO HLA MOLECULES, AND THE USE THEREOF, which is hereby incorporated by reference in its entirety. The use of analogs incorporating non-standard amino acids with a primary goal of improving biochemical stability is reported by Blanchet, J.-S. et al., *J. Immunol.* 167:5852-5861, 2001, which is hereby incorporated by reference in its entirety.

SSX-2 41-49 Analogs

[0131] As noted above, the natural immune response to SSX-2 in cancer patients, including the response to SSX-2_{41-49}, may not be effective in controlling cancer. Additionally, wild-type SSX-2_{41-49} is only a moderately immunogenic peptide that can further limit its clinical potential. Stronger SSX-2 specific immune responses induced by the use of superagonist analogs results in clinical benefits for patients with SSX-2 positive tumor.

[0132] Thus, in one embodiment, the analogs can be used in compositions to stimulate the immune response of a subject to mount an immune response against a target cell displaying the target antigen. The embodiment is contemplated to have utility in the treatment and prevention of neoplastic and viral disease.

[0133] Since the wild-type SSX-2_{41-49} is only a moderately immunogenic peptide that may prevent it from eliminating tumors effectively *in vivo*, a method was used to *de novo* design SSX-2_{41-49} variants that were more potent or had a variety of improved properties. By using a more immunogenic SSX-2 analog peptide, it was possible to stimulate a stronger immune response and/or to amplify the naturally occurring immune response to achieve a better chance of clinical response. Thus, the binding properties (affinity and HLA-A*0201/peptide complexes stability), immunogenicity, antigenicity and cross-reactivity to the wild-type epitope were analyzed for each of the analogs to identify an improved property. In some embodiments, by improved property it is meant generally, that the analog can be better used for some purpose than the wild-type. Thus, the analog need not exhibit improved binding, stability, or activity to be improved and may even show a reduced ability to mediate certain parts of the process, but still be improved for use in another way. For example, analogs that retain some activity, but not all activity may be better in human systems that are tolerized to the wild-type antigen.

[0134] Previously, modifications of natural tumor-associated peptide epitopes by incorporating favorable anchor residues have generated analogs with improved binding profiles with HLA molecules and enhanced immunogenicity. One of the most successful examples is the A27L peptide analog of Melan-A 26-35 epitope. Valmori et al., "Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogs," *J Immunol.* 1998, 160(4): 1750-8; which is hereby incorporated by reference in its entirety. The original epitope failed to form a stable complex with HLA-A2 molecules since it lacks optimum anchor amino acid residue at position 2. The modified A27L Melan A 26-35 peptide analog has demonstrated

unequivocally increased binding profiles with HLA-A2 molecules and greater immunogenicity than its wild-type counterpart. Immunizing patients with this analog can generate strong T cell immune responses that were able to recognize the wild-type epitope presented at the cell surfaces. Similar modifications have been obtained successfully with many other tumor-associated epitopes such as GP100 209-217 (Parkhurst et al., "Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues," *J Immunol.* 1996, 157(6): 2539-48; which is hereby incorporated by reference in its entirety), Her-2 369-377 (Vertuani et al., "Improved immunogenicity of an immunodominant epitope of the HER-2/neu protooncogene by alterations of MHC contact residues," *J Immunol.* 2004, 172(6): 3501-8; which is hereby incorporated by reference in its entirety).

[0135] Up to this point no SSX-2_{41-49} analogs have been designed and studied even though they hold great promise for development of SSX-2-based vaccines to treat a variety of cancers, particularly SSX-2 positive cancers and/or tumors. Thus, methods are disclosed herein that can be used for the identification and production of analogs to a Synovial sarcoma X breakpoint 2 (SSX-2) wild-type sequence. Using the methods, a panel of 95 novel SSX-241-49 analogs based on the wild-type sequence from amino acids 241-249 were identified with a variety of improved properties. The improved properties include, but are not limited to, binding to class I MHC and T cell receptor (TCR) molecules, and biological responses such as IFN-γ secretion, cytotoxicity, and tumor cell lysis. Peptides with improved potency that retained cross-reactivity with the wild-type epitope were identified. Among these analogs, some have been demonstrated to be the superagonist variants of the wild-type SSX-241-49 peptide, some of which analogs have been shown to have much higher affinity with HLA-A*0201 molecule, and the peptide-HLA complex possessed extended stability. When the mice were immunized with these analogs, they were able to induce enhanced CTL immune responses in HHD transgenic mice. The resulting CTLs could effectively lyse A2+ and SSX-2+ tumor cell lines both *in vivo* and *in vitro*, which indicated that the CTLs generated using the analogs were able to recognize the wild-type SSX-241-49 epitope that naturally presented at the cell surfaces. In comparison with the wild-type SSX-241-49 epitope, the analogs are better candidates for the development of cancer vaccines.

[0136] Embodiments include families of one or more peptides of 9 or 10 amino acids in length related by sequence to amino acids 41-49 of the human cancer testis (CT) antigen SSX-2 (SSX-2_{41-49}). The individual peptide embodiments have one to several defined amino acid substitutions in the wild-type sequence. The substituted amino acids are, variously, other members of the standard set of amino acids commonly genetically encoded, derivatives thereof, their D-stereoisomers, or other non-standard L-amino acids. These analogs are useful for investigating the interaction of the wild-type epitope with class I MHC and TCR molecules and other components of the immune response, and for designing additional analogs with further optimized immunologic properties. Some embodiments of the analogs have at least similar immunologic properties to the

wild-type epitope in the HLA-transgenic mouse model in which they have been tested. Such peptides can be useful in humans, as SSX-2 is a self-antigen to which a degree of tolerance may be expected, and the amino acid differences of the analogs can help to stimulate populations of T cells that have avoided negative selection but are cross-reactive with the wild-type epitope. Various peptide embodiments can have one or more improved immunologic properties in that they possess greater affinity for MHC or greater stability of binding to MHC, elicit greater cytokine production or require lower peptide concentrations to elicit similar cytokine production from T cells that recognize the wild-type epitope, are more immunogenic, can induce or amplify a cross-reactive cytolytic response to the wild-type epitope, or can break tolerance.

[0137] In one embodiment, the analogs can have at least one substitution at a residue selected from the group consisting of, P1, P2, P4, P6, P8, P9 and P10. In a further embodiment, the analogs can have at least two substitutions at residues selected from the group consisting of: P1, P2, P4, P6, P8, P9 and P10. In a further embodiment, the analogs can have at least three substitutions at residues selected from the group consisting of: P1, P2, P4, P6, P8, P9 and P10. In a further embodiment, the analogs can have substitutions at positions P2 and P9. In a further embodiment, the peptides can have substitutions at residues P1, P2, and P9. In a further embodiment, the peptide analogs can have substitutions at residues P1, P2, and P4. In a further embodiment, the peptide analogs can have substitutions at residues P1, P2, and P6. In a further embodiment, the peptide analogs can have substitutions at residues P1, P2, and P8. In one embodiment, two substitutions can produce improved properties. In a further embodiment, one substitution can produce improved properties. In a further embodiment, three substitutions can produce improved properties. In a further embodiment, the one or more substitutions can produce improved properties but are still recognized by a TCR that recognizes the wild-type sequence (still cross-react with the wild-type sequence).

[0138] One embodiment relates to epitope arrays and other polypeptides comprising the epitope analog sequences that can be processed to liberate the analog. Further embodiments relate to nucleic acids, particularly DNA plasmids, encoding such polypeptides, or simply an analog, and their expression therefrom. The analogs, the polypeptides comprising them, and the encoding nucleic acids can all be components of immunogenic compositions, particularly compositions suitable for intralymphatic delivery, that constitute further embodiments.

Analog design

[0139] Embodiments relate to the SSX-2_{41-49} peptide which contain substitutions of the sequence KASEKIFYV (See Figure 1). In a further embodiment, the analog can be generally an analog of the SSX-2_{41-50} decamer peptide with the sequence KASEKIFYVY. The residues or amino acids that make up the peptide are referred to herein as P1-P9 or P1-P10 to designate the position within the peptide as numbered from the N- to the C-terminus, P1 corresponding to the N-terminal

Lysine and P9 corresponding to the C-terminal Valine in the nonamer. Alternatively, the residues may be referred to by the primary activity of the molecule that they are involved in. For example, residue P2 is described as the N-terminal primary anchor molecule, while P9 (or P10 in the decamer) is described as the primary C-terminal anchor. Residues P4, P6 and P8 are primarily involved in TCR interactions. Substitutions can use any amino acids, including standard and non-standard amino acids, known to one of skill in the art. A number of exemplary amino acids are disclosed herein, however, the substitutions disclosed herein are not meant to be a list that includes all imagined substitutions, but are exemplary of the substitutions that are possible. One of skill in the art may find a number of other non-standard amino acids in catalogs and references that may be purchased or chemically produced for use with the analogs herein.

[0140] A number of possible analogs were produced by modification of peptide anchor residues to achieve better HLA binding profiles and higher immune responses, including at the N-terminal primary anchor (P2 position), at the N-terminal secondary anchor (P1 position), at the N-terminal primary and secondary anchor (P1 and P2 positions), and at the N-terminal primary/secondary anchor (P1 and P2 positions) and C-terminal primary anchor (P9 position). Further, peptides with modifications at the anchor residues and TCR contact residues were produced to circumvent T cell tolerance for self-antigens, these modifications included modifications at the N-terminal primary/secondary anchor (P1 and P2 positions) and secondary TCR recognition sites (P4, P6 and/or P8 positions), modifications at the N-terminal primary/secondary anchors (P1 and P2 position), and modifications at the C-terminal primary anchor (P9) and at secondary TCR recognition sites (P4, P6 and/or P8 positions). Further, decamer analogs were produced.

[0141] The choice of which residues would best produce analogs with improved properties involved analysis of studies of MHC peptide interactions, studies of TCR peptide interactions and previous analogs that were known in the art. Some residues are primarily involved in a specific interaction and some are secondarily or even tertiarily involved. Thus, the knowledge of how the residues are involved in the binding to these molecules was involved in the analysis. Further some of the wild-type residues are preferred, meaning that they work well for the intended interaction, while others are non-preferred, meaning that they work poorly for the interaction. Thus, in one embodiment, the non-preferred residues can be substituted. For example, the valine at the C-terminus is generally a preferred anchor residue because it produces a strong interaction with the HLA molecule and, thus, it was less preferred to substitute this residue. However, modifications of wild-type tumor-associated peptide epitopes by incorporating favorable anchor residues have generated analogs with improved binding profiles with HLA molecules and enhanced immunogenicity. One of the most successful examples is the A27L peptide analog of Melan-A 26-35 epitope (Valmori D, et al. J Immunol. 160(4): 1750-8, 1998; which is hereby incorporated by reference in its entirety). The original epitope failed to form a stable complex with HLA-A2 molecules since it lacked an optimal anchor residue at position 2. In contrast the modified Melan A_{26-}

35 A27L peptide analog demonstrated unequivocally increased binding profiles with HLA-A2 molecules and greater immunogenicity than its wild-type counterpart. Immunizing patients with this analog generated strong T cell immune responses that were able to recognize the wild-type epitope presented at the cell surfaces. Similar modifications were obtained successfully with many other tumor-associated epitopes such as GP100 209-217 (Parkhurst MR, et al. J Immunol. 157(6): 2539-48, 1996; which is hereby incorporated by reference in its entirety), Her-2 369-377 (Vertuani S, et al. J Immunol. 172(6): 3501-8, 2004; which is hereby incorporated by reference in its entirety).

[0142] The choice of how many residues to substitute involves a desire to substitute better residues while still retaining enough of the qualities of the epitope that it will still be recognized by T cells which recognize the wild-type epitope. Thus, in one embodiment, one or two substitutions can be made to the wild-type peptide. In a further embodiment, more than two substitutions can be made to the wild-type peptide, while still retaining cross-reactivity with the wild-type peptide.

[0143] Thus, generally, the part of the peptide that is involved in TCR recognition is desirably substituted to produce improved immunogenicity while still cross-reacting with the wild-type epitope. For example, a peptide that shows increased immunogenicity is preferred. Because the P2 or second amino acid at the N-terminal end is believed to be primarily involved in the process of producing improved immunogenicity, primarily through improved binding properties, it is a preferred substitution site and a number of modifications were made in the exemplary analogs to identify desired substitutions. Similar considerations apply to the carboxy-terminal position, PΩ which also can be important for MHC binding.

[0144] Thus, in one embodiment, the analog can include a substitution at the P2 residue that substitutes a more hydrophobic residue for the wild-type alanine. In a further embodiment, the hydrophobic residue also can possess a more bulky side chain. In a further embodiment, the residue at P1 can be substituted with a more hydrophobic residue. In a further embodiment, residues P1 and P2 both can be substituted with more hydrophobic residues. In further embodiments at least one residue at P1, P2, and P9 can be substituted. In a further embodiment, at least two residues at P1, P2 and P9 can be substituted. In a further embodiment at least two residues at P1, P2, P9 P4 and P6 can be substituted including one or more residues involved in TCR binding.

[0145] Further, substitutions of those residues only secondarily involved in binding to TCR or the MHC molecule can be advantageous. For example, substitution of secondary TCR binding amino acids can generate analogs that still bind and produce a response and do not interfere with the binding to the MHC molecule, but preferably overcome the tolerance issues of self-antigens. This is useful because a patient who has cancer may be partially tolerized to the antigen. Thus, in order to overcome that tolerance, an analog that retains some activity can be preferable to an analog with more improved immunogenicity, because it will be less likely to be recognized as "self" by the immune system.

1. N-terminal proximal primary anchor modification(P2)

[0146] The N-terminal primary anchor is the 2nd N-terminal amino acid of the peptide and is the N-terminal proximal primary anchor. It is primarily involved in the interaction with the MHC molecule and substitutions can result in improved binding and stability. However, it may be secondarily involved in TCR interactions also. Thus, substitutions at this site can result in a peptide with improved interaction with MHC molecules as well as improved interaction with the TCR.

[0147] The alanine found at this position in the wild-type sequence is generally believed to be non-preferred for the interaction with the MHC molecule. Thus, preferred embodiments of the analogs have a substitution at this position. In one embodiment, the original Ala 42 found in the wild-type sequence can be substituted with a more hydrophobic amino acid. Any more hydrophobic amino acid may be used including any that is available or known to one of skill in the art, including standard amino acids and non-standard amino acids. In a further embodiment, the original Ala 42 is substituted with a more hydrophobic amino acid also possessing a bulky side chain. Examples of more hydrophobic amino acids includes, but are not limited to: Leu, Val, Ile, Met, α-aminobutyric acid, Norleucine and Norvaline.

TABLE 1

N-TERMINAL PROXIMAL PRIMARY ANCHOR MODIFICATION

Catergory	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (mM)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs) *
Native peptide	SSX2 41-49	KASEKIFYV	22 / 1017	14.64	1.0	11	1
N-terminal Primary Anchor	SSX2 41-49 (A42L)	KLSEKIFYV	28 / 73228	8.89	1.6	19	0.03
	SSX2 41-49 (A42V)	KVSEKIFYV	22 / 6407	5.2	2.8	20	0.03
	SSX2 41-49 (A42I)	KISEKIFYV	26 / 10068	8.8	1.7	22.5	3
	SSX2 41-49 (A42M)	KMSEKIFYV	26 / 52887	8.8	1.7	22.5	0.1
	SSX2 41-49 (A42(D-Ala))	K(D-Ala)SEKIFYV	NA	N/B	N/B	N/B	10
	SSX2 41-49 (A42(D-Leu))	K(D-Leu)SEKIFYV	NA	N/B	N/B	N/B	N/T
	SSX2 41-49 (A42(D-Val))	K(D-Val)SEKIFYV	NA	N/B	N/B	N/B	3
	SSX2 41-49 (A42(Nal-1))	KNal-1SEKIFYV	NA	N/B	N/B	N/B	>10
	SSX2 41-49 (A42(Nal-2))	KNal-2SEKIFYV	NA	13.9	1.1	N/A	3
	SSX2 41-49 (A42(Abu))	KAbuSEKIFYV	NA	7.56	1.9	N/A	0.3
	SSX2 41-49 (A42(Nle))	KNleSEKIFYV	NA	5.82	2.5	24	0.1
	SSX2 41-49 (A42(Nva))	KNvaSEKIFYV	NA	11.4	1.3	N/A	0.1
	SSX2 41-49 (A42(Aib))	KAibSEKIFYV	NA	18.4	0.8	N/A	3

2. N-terminal secondary anchor modification (P1)

[0148] The N-terminal secondary anchor is the first amino acid at the N-terminus. This residue is Lys 41 and is defined as a secondary anchor residue in interacting with the HLA-A*0201 molecule. However, it is also engaged in the interaction with the T cell receptors to a certain degree. Therefore, modifications of this position can generate some heteroclitic analogs that are more immunogenic and more suitable for the development of tumor vaccines. Although the lysine at this position is generally considered to be favored, substitutions can result in highly improved properties.

[0149] Thus, in one embodiment, the original Lys 43 found in the wild-type sequence can be substituted with a more hydrophobic amino acid. Any more hydrophobic amino acid can be used, including any that is available or known to one of skill in the art, including standard amino acids and non-standard amino acids. In a further embodiment, the Lys 43 can be substituted with an

aromatic amino acid. Examples of more hydrophobic amino acids include, but are not limited to: Phe, Tyr, Trp, and D-Lys.

Table 2

N-TERMINAL SECONDARY ANCHOR MODIFICATIONS

Category	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (mM)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs) *
Native	SSX2 41-49	KASEKIFYV	22 / 1017	14.64	1.0	11	1
N-terminal Secondary Anchor	SSX2 41-49 (K41F)	FASEKIFYV	23 / 1336	9.55	1.5	>24	0.3
	SSX2 41-49 (K41W)	WASEKIFYV	22 / 1336	27.07	0.5	N/A	>10
	SSX2 41-49 (K41Y)	YASEKIFYV	21 / 1336	8.74	1.7	>24	3
	SSX2 41-49(K41(D-Lys))	**(D-Lys)**ASEKIFYV	NA	N/B	N/B	N/B	>10
	SSX2 41-49 (K41(Phg))	**Phg**ASEKIFYV	NA	5.83	2.5	>24	0.1
	SSX2 41-49 (K41(Cha))	**Cha**ASEKIFYV	NA	N/B	N/B	N/B	>10
	SSX2 41-49 (K41(Phe-4F))	**Phe(4-F)**ASEKIFYV	NA	6.72	2.2	>24	3
	SSX2 41-49 (K41(Phe-4NO2))	**Phe(4-NO2)**ASEKIFYV	NA	12.8	1.1	N/A	3
	SSX2 41-49 (K41(O-methyl Tyr))	**O-methyl-Tyr**ASEKIFYV	NA	19.5	0.8	20	3
	SSX2 41-49 (K41(b-(3-benzothienyl)Ala))	**b-(3-benzothienyl)Ala**ASEKIFYV	NA	24.1	0.6	N/A	10

3. N-terminal primary and secondary modifications (P2 and P1)

[0150] In one embodiment, both primary and secondary anchor residues were substituted to result in improved binding affinity to the HLA molecule. In a further embodiment, the double substitution produced improved stability of binding to the HLA molecule. In further embodiments, the binding and/or stability was not improved and may have even been reduced, but other properties of the molecule were improved, such as activity or recognition by a tolerized individual.

Table 3

N-TERMINAL PRIMARY AND SECONDARY ANCHOR MODIFICATION

Catergory	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (mM)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs) *
Native	SSX2 41-49	KASEKIFYV	22 / 1017	14.64	1.0	11	1
N-terminal Primary/Secondary Anchor	SSX2 41-49 (K41Y, A42L)	YLSEKIFYV	29 / 96243	11.8	1.2	>24	N/T
	SSX2 41-49 (K41Y, A42V)	YVSEKIFYV	23 / 8421	14.6	1.0	>24	0.1
	SSX2 41-49 (K41Y, A42M)	YMSEKIFYV	27 / 69508	25	0.6	>24	3
	SSX2 41-49 (K41Y, A42I)	YISEKIFYV	27 / 13233	6.5	2.3	N/A	1
	SSX2 41-49 (K41F, A42L)	FLSEKIFYV	28 / 96243	4.9	3.0	>24	0.3
	SSX2 41-49 (K41F, A42V)	FVSEKIFYV	22 / 8421	4.675	3.1	24	0.1
	SSX2 41-49 (K41F, A42M)	FMSEKIFYV	26 / 69508	6.58	2.2	>24	3
	SSX2 41-49 (K41F, A42I)	FISEKIFYV	26 / 13233	5.368	2.7	>24	0.3
	SSX2 41-49 (K41W, A42L)	WLSEKIFYV	27 / 96243	4.472	3.3	>24	0.3
	SSX2 41-49 (K41W, A42V)	WVSEKIFYV	21 / 8421	4.82	3.0	>24	1
	SSX2 41-49 (K41W, A42M)	WMSEKIFYV	25 / 69508	5.13	2.9	>24	1
	SSX2 41-49 (K41W, A42I)	WISEKIFYV	25 / 13233	6.98	2.1	>24	0.1
	SSX2 41-49 (K41(D-Lys), A42L)	(D-Lys)LSEKIFYV	N/A	2.5	5.9	15	10
	SSX2 41-49 (K41(D-Lys), A42V)	(D-Lys)VSEKIFYV	N/A	24.5	0.6	N/A	10

4. N-terminal primary/secondary anchor and C-terminal primary modification (P2, P1 and P9)

[0151] The C-terminal Val of the wild-type peptide is generally a preferred anchor residue and primarily involved in the interaction with the MHC molecule. However, substitutions were carried out to identify which amino acids improve the analogs having primary and secondary N-terminal modifications. These C-terminal substitutions can be used in the absence of one or more N-terminal modifications also.

[0152] These modifications were shown to improve binding affinity and stability and in some cases resulted in analogs with decreased cross-reactivity. Thus, in some embodiments, the substitution to the C-terminus resulted in a peptide with improved binding and/or stability without decreased cross-reactivity. However, in other embodiments the substitution to the C-terminus resulted in a peptide with improved binding and/or stability with equal or decreased cross-reactivity. Each of the molecules can be of use in certain cases or in certain patients. In one embodiment, the valine at the C-terminus is substituted with a large aliphatic amino acid.

Table 4

N-TERMINAL PRIMARY/SECONDARY ANCHOR AND C-TERMINAL PRIMARY MODIFICATIONS

Catergory	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (ΩM)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs) *
Native	SSX2 41-49	KASEKIFYV	22 / 1017	14.64	1.0	11	1
N-terminal Primary/Secondary Anchor, C-terminal Primary Anchor	SSX2 41-49 (K41F, A42V, V49L)	**FV**SEKIFY**L**	22 / 2586	10.7	1.4	17	>10
	SSX2 41-49 (K41F, A42V, V49I)	**FV**SEKIFY**I**	20 / 1263	9	1.6	24	0.3
	SSX2 41-49 (K41F, A42V, V49A)	**FV**SEKIFY**A**	16 / 601	6.9	2.1	16	1
	SSX2 41-49 (K41F, A42V, V49M)	**FV**SEKIFY**M**	16 / 601	17.8	0.8	22	>10
	SSX2 41-49 (K41F, A42V, V49Nle)	**FV**SEKIFY**(Nle)**	N/A	5.59	2.6	>24	>10
	SSX2 41-49 (K41F, A42V, V49Nva)	**FV**SEKIFY**(Nva)**	N/A	1.89	7.7	20	0.1
	SSX2 41-49 (K41F, A42V, V49MeVal)	**FV**SEKIFY**(MeVal)**	N/A	17.9	0.8	22	10
	SSX2 41-49 (K41F, A42V, V49Aib)	**FV**SEKIFY**(Aib)**	N/A	N/A	N/A	N/A	>10
	SSX2 41-49 (K41F, A42V, V49Abu)	**FV**SEKIFY**(Abu)**	N/A	3.43	4.3	20	1
N-terminal Primary Anchor, C-terminal Primary Anchor	**SSX2 41-49 (A42V, V49I)**	KVSEKIFYI	20 / 961	13.9	1.1	N/A	0.3
	SSX2 41-49 (A42L, V49I)	KLSEKIFYI	26 / 10984	5.682	2.6	N/A	0.03
	SSX2 41-49 (A42a, V49v)	K**(D-Ala)**SEKIFY**(D-Val)**	N/A	N/B	N/B	N/B	>10
C-terminal Primary Anchor	SSX2 41-49 (V49I)	KASEKIFYI	20 / 152.56	14	1.0	N/A	10

5. N-terminal primary/secondary anchor and TCR residues modification

[0153] The TCR sites are generally recognized as residues P4, P6, and P8 and are the primary residues involved in the binding to the TCR. However, other residues may also be involved in the interaction to a lesser extent. In one embodiment, one or more of the sites primarily involved in TCR interaction can be substituted to increase the interaction. Preferably, these substitutions can generate heteroclitic analogs that do not interfere with binding to the MHC molecule, but overcome the tolerance issues of the wild-type peptides. In a further embodiment, at least one TCR substitution

can be included with at least one substitution at position P1, P2, and/or P9. In a further embodiment, the substitution at any one or more of the P4, P6, and P8 positions can be a polar amino acid. In a further embodiment, the substitution can be an aromatic amino acid at position P8. In a further embodiment, the substitution can be an amino acid with a large aliphatic side chain at position P6. In a further embodiment, the substitution can be an amino acid which has a larger side chain to preserve the interaction.

Table 5

N-TERMINAL PRIMARY/SECONDARY ANCHOR AND TCR SITES MODIFICATION

Catergory	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (mM)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs) *
Native	**SSX2 41-49**	KASEKIFYV	22 / 1017	14.64	1.0	11	1
N-terminal Primary/Secondary Anchor, TCR sites	SSX2 41-49 (K41F, A42V, E44D)	**FVSD**KIFYV	21/ 8421	13.18	1.1	N/A	>10
	SSX2 41-49 (K41F, A42V, E44N)	**FVSN**KIFYV	20 / 2054	8.97	1.6	N/A	>10
	SSX2 41-49 (K41F, A42V, E44S)	**FVSS**KIFYV	20 / 2054	17.5	0.8	N/A	>10
	SSX2 41-49 (K41F, A42V, E44T)	**FVST**KIFYV	20 / 2054	12.94	1.1	N/A	>10
	SSX2 41-49 (K41F, A42V, E44Q)	**FVSQ**KIFYV	20 / 2054	40.8	0.4	N/A	10
	SSX2 41-49 (K41F, A42V, E44Nle)	**FVS(Nle)**KIFYV	N/A	13	1.1	N/A	10
	SSX2 41-49 (K41F, A42V, E44Nva)	**FVS(Nva)**KIFYV	N/A	3.8	3.9	>24	3
	SSX2 41-49 (K41F, A42V, I46L)	**FVSEKL**FYV	22 / 8421	7.8	1.9	24	3
	SSX2 41-49 (K41F, A42V, I46V)	**FVSEKV**FYV	22 / 8421	**N/A**	**N/A**	24	1
	SSX2 41-49 (K41F, A42V, I46M)	**FVSEKM**FYV	18 / 8421	9.2	1.6	22	>10
	SSX2 41-49 (K41F, A42V, I46Nle)	**FVSEK(Nle)**FYV	N/A	12.8	1.1	19	10
	SSX2 41-49 (K41F, A42V, I46Nva)	**FVSEK(Nva)**FYV	N/A	6.21	2.4	>24	1
	SSX2 41-49 (K41F, A42V, Y48T)	**FVSEKIFT**V	24 / 1531	3.9	3.8	24	>10
	SSX2 41-49 (K41F, A42V, Y48F)	**FVSEKIFF**V	22 / 8421	8.8	1.7	20	10
	SSX2 41-49 (K41F, A42V, Y48S)	**FVSEKIFS**V	24 / 1531	3.8	3.9	20	>10
	SSX2 41-49 (K41F, A42V, Y48(Phe-4F))	**FVSEKIF (Phe-4F)**V	N/A	10.6	1.4	24	10
	SSX2 41-49 (K41F, A42V, Y48Phg)	**FVSEKIF (Phg)**V	N/A	5.85	2.5	>24	>10
	SSX2 41-49 (K41F, A42V, I46L,Y48T)	**FVSEKLFT**V	24 / 1531	5.67	2.6	24	>10
	SSX2 41-49 (K41F, A42V, I46L,Y48S)	**FVSEKLFS**V	24 / 1531	**N/A**	**N/A**	N/A	N/T

Catergory	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (mM)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs)*
N-terminal Primary/Secondary Anchor, C-terminal Primary Anchor, TCR sites	SSX2 41-49 (K41F, A42V, I46L,Y48T, V49A)	FVSEKLFTA	18 /109	6.3	2.3	12	>10
	SSX2 41-49 (K41F, A42V, I46L,Y48S, V49A)	FVSEKLFSA	18 /109	6.2	2.4	N/A	>10

6. C-terminal amide

[0154] In some embodiments, the C-terminal residue can be modified to contain an amide in the place of the free carboxylic acid. Thus, for example, if the peptide is a 9-mer (nonamer) the P9 residue can be modified. If the peptide is a 10-mer (decamer) the P10 residue can be modified. Preferably this results in a peptide or analog that has increased stability in biological media, including but not limited to blood, lymph, and CNS. Preferably, the peptides can retain the other necessary activities to result in an analog usable for vaccination or as an immunogen.

Table 6

C-TERMINAL AMIDE

Catergory	Peptide name	Sequence	Predictive Scores (R/NIH)	Half-maximal Binding (□M)	Relative affinity (1/RA)	Stability (T1/2) (Hrs)	Cross-reactivity and fct avidity (native to analogs)*
Native	SSX2 41-49	KASEKIFYV	22 / 1017	14.64	1.0	11	1
C-terminal amide	SSX2 41-49-NH2	KASEKIFYV-NH2	N/A	N/B	N/B	N/T	>10
	SSX2 41-49-NH2 (A42L)	KLSEKIFYV-NH2	N/A	N/B	N/B	N/T	3
	SSX2 41-49-NH2 (A42V)	KVSEKIFYV-NH2	N/A	N/B	N/B	N/T	10

7. Decamers

[0155] The length of typical MHC binding peptides can vary from about 8 to about 11 amino acids in length. However, most of the previously used HLA-A*0201 are 9-mers (nonamers) or 10-mers (decamers). Thus, in one embodiment, the analog can be an analog of the wild-type sequence SSX-2_{41-50}. However, because the wild-type 10-mer does not have the correct binding

motif and showed no immunological activity, a 10-mer was created by substituting amino acids at the P10 position and identifying the effect of various wild-type and analogs (see Figure 1).

8. Remaining residues

[0156] With reference to Figures 1A and 1B, any residues can also be substituted with conservative amino acids. Conservative substitutions can be paired with any of the above substitutions that can produce an effect. Alternatively, conservative substitutions can be specifically at residues that are not believed to be involved in any of the activities at a primary, secondary, or even tertiary level. Such residues include P3, P5 and P7. For example, the Serine at position P3 can be substituted with an alanine or threonine to produce an analog. Typically, such conservative substitutions do not significantly affect the activity of the analog, however, in some embodiments they can increase certain activities or decrease certain activities.

NY-ESO-$1_{157-165}$ Analogs

[0157] Many features regarding a variety of embodiments and aspects of analog design are disclosed above, either generally or as applied to the SSX-2 epitope. It is to be understood that such disclosure is also applicable to this and subsequent epitopes. Explicit restatement of such disclosure will be minimized for the sake of brevity.

[0158] Embodiments relate to analogs of the MHC class I-restricted T cell epitope NY-ESO-$1_{157-165}$, SLLMWITQC (SEQ ID NO. 1), polypeptides comprising these analogs that can be processed by pAPC to present the epitope analogs, and nucleic acids that express the analogs. The analogs can have similar or improved immunological properties compared to the wild-type epitope.

[0159] One embodiment relates to methods to derivatize and improve analogs of NY-ESO-$1_{157-165}$, along with specific sequences that encompass substitutions. The analogs can contain at least one substitution, but can have multiple substitutions comprising standard or non-standard amino acids singly or in various combinations. The analogs can result in peptides with retained or improved properties.

[0160] The epitope NY-ESO-$1_{157-165}$ has been shown to be presented by NY-ESO-1 expressing cell lines, by measuring the epitope specific T cell activity against such cells (Jaeger, E. et al., J. Exp. Med. 187:265-270, 1998; U.S. Patent No. 6,274,145 entitled ISOLATED NUCLEIC ACID MOLECULE ENCODING CANCER ASSOCIATED ANTIGEN, THE ANTIGEN ITSELF, AND USES THEREOF), which is incorporated herein by reference in its entirety. Methodologies to improve the physico-chemical properties of the peptide NY-ESO-$1_{157-165}$ have been described (U.S. Patent No. 6,417,165 entitled NY-ESO-1-PEPTIDE DERIVATIVES, AND USES THEREOF), which is incorporated herein by reference in its entirety, and can consist of replacement of the terminal cysteine with other amino acids that preserve or enhance the interaction with MHC and are devoid of the deleterious property of disulfide C-C bond formation interfering with the activity.

However, sole manipulation of the C terminal cysteine residue ignores the advantages of optimizing multiple residues throughout the peptide for major histocompatibility (MHC) and/or T cell Receptor (TCR) binding. Thus, beyond the practicality of mutating the Cys residue, there is considerable opportunity in mutating additional amino acids throughout the peptide. For example, substitutions can be used to further optimize the binding to MHC and /or TCR in a fashion that enables more effective application in clinics.

[0161] Embodiments relate to families of one or more peptides of 9 or 10 amino acids in length related by sequence to amino acids 157-165 of the human cancer testis (CT) antigen NY-ESO-1 (NY-ESO-$1_{157-165}$).

Analog design

[0162] The analog is generally an analog of the NY-ESO-$1_{157-165}$, with the sequence SLLMWITQC (SEQ ID NO:1). Analysis of whether wild-type amino acids are preferred or non-preferred used previous analyses of other peptide-MHC or TCR interactions. For example, the Cysteine at the C-terminus is generally a non-preferred anchor residue because it does not produce a strong interaction with the HLA molecule and, thus, it was highly preferred to substitute this residue. However, although the Serine at position P1 is generally preferred, it was found that substituting an aromatic could produce a peptide with improved properties. Further the Leucine at position P2 is generally acceptable, but substituting a hydrophobic and/or bulky amino acid resulted in a peptide with improved properties. The residues which are primarily involved in the interaction with the TCR (P4, P6 and P8) showed a preference generally for some polarity, and in the case of P8 an aromatic generally produced peptides with favorable properties.

[0163] One preferred embodiment relates to an analog that has a substitution at the P2 position. Preferably, the substitution can be a hydrophobic residue. More preferably, the substitution can be a bulky hydrophobic residue. In a further embodiment, the residue at P1 can be substituted with a more hydrophobic residue. In a further embodiment, residues P1 and P2 can be both substituted with more hydrophobic residues. In further embodiments at least one residue at P1, P2, and P9 can be substituted. In a further embodiment, at least two residues at P1, P2 and P9 can be substituted. In a further embodiment at least two residues at P1, P2, P9, P4, and P6 can be substituted, including one or more residues involved in TCR binding. In a further embodiment, the residue at P8 can be substituted with an aromatic. Examples of the following substitutions are shown in Figures 13A-13C.

1. N-terminal proximal primary anchor modification(P2)

[0164] The N-terminal primary anchor is the 2nd N-terminal amino acid of the peptide, thus, it is the N-terminal proximal primary anchor. Although the original Leucine 158 is not considered "non-preferred" for binding to the MHC molecule, substitutions can produce a peptide

with improved binding. Thus, in one embodiment, the original Leu 158 found in the wild-type sequence can be substituted with a similarly or more hydrophobic amino acid. Any hydrophobic amino acid may be used, including one that is available to or that is known to one of skill in the art, including standard amino acids and non-standard amino acids. In a further embodiment, the original Leu 158 can be substituted with a more hydrophobic amino acid also possessing a bulky side chain. Examples of more hydrophobic amino acids include, but are not limited to: Leu, Val, Ile, Met, α-aminobutyric acid, Norleucine and Norvaline. Further, a naphthal side chain can also be substituted. Preferably, the substitution results in improved binding and stability with the HLA molecule. However, this residue may be secondarily or tertiarily involved in TCR interactions, and substitutions may also result in improved recognition by the TCR.

2. N-terminal secondary anchor modification (P1)

[0165] The N-terminal secondary anchor is the first amino acid at the N-terminus or P1. This residue is involved in a number of interactions. The residue of Ser 157 was defined as a secondary anchor residue in interacting with HLA-A*0201 molecule, it also engaged in the interaction with the T cell receptors to a certain degree. Therefore, modifications of this position generate some heteroclitic analogs that are more immunogenic and more suitable for the development of tumor vaccines. Thus, substitutions can result in a variety of improved qualities.

[0166] Although the Serine is not considered "non-preferred," a number of substitutions can result in improved qualities of the peptide. Thus, in one embodiment, the original Ser 157 found in the wild-type sequence can be substituted with a more hydrophobic amino acid. Any more hydrophobic amino acid can be used, including one that is available to or that is known to one of skill in the art, including standard amino acids and non-standard amino acids. Examples of more hydrophobic amino acids include, but are not limited to: Phe, Tyr, Trp, and D-Lys.

3. N-terminal primary and secondary modifications (P2 and P1)

[0167] In one embodiment, both primary and secondary anchor residues were substituted to result in improved binding affinity to the HLA molecule. In a further embodiment, the double substitution produced improved stability of binding to the HLA molecule. In further embodiments, the binding and/or stability was not improved and may have even been reduced, but other properties of the molecule were improved, such as activity or recognition by a tolerized individual.

4. N-terminal primary/secondary anchor and C-terminal primary modification (P2, P1 and P9)

[0168] The C-terminal cysteine of the wild-type peptide is generally a non-preferred anchor residue. Because this residue is generally primarily involved in the interaction with the MHC molecule, it can be preferred to substitute residues that result in a stronger interaction with the MHC molecule. Thus, substitutions were shown to improve binding affinity and stability and in some cases resulted in analogs with decreased cross-reactivity. In some embodiments, the substitution to the C-terminus can result in a peptide with improved binding and/or stability without decreased cross-reactivity. However, in other embodiments the substitution to the C-terminus can result in a peptide with improved binding and/or stability with equal or decreased cross-reactivity. Because substitution of this residue have been previously shown to provide improved peptides, it can be preferable produce peptides that were more improved in the interaction with the MHC molecule as well as other interactions, such as the recognition by the TCR. Thus, in some embodiments, the C-terminal substitution can be paired with at least one other substitution. Examples of amino acid substitutions to the C-terminus include, but are not limited to, valine, lysine, alanine, and isoleucine.

5. N-terminal primary/secondary anchor and TCR residue modifications

[0169] The primary residues involved in the interaction with the TCR are generally recognized as residues P4, P6, and P8. However, other residues may also be involved in the interaction to a lesser extent. In one embodiment, one or more of the sites primarily involved in TCR interaction can be substituted to result in an improved interaction. Preferably, these substitutions generate heteroclitic analogs that do not interfere with binding to the MHC molecule, but overcome the tolerance issues of the wild-type peptides. In one embodiment, at least one TCR substitution can be included with at least one substitution at position P1, P2, and/or P9. In one embodiment, amino acids with some polarity can be substituted at P4, P6, and P8. In a further embodiment, amino acids which are aromatic can be substituted at the P8 position.

6. C-terminal amide

[0170] In some embodiments, the C-terminal residue can be modified to contain an amide in the place of the free carboxylic acid. Thus, for example if the peptide is a 9-mer (nonamer) the P9 residue can be modified. If the peptide is a 10-mer (decamer) the P10 residue can be modified. Preferably this results in a peptide or analog that has increased stability in biological media, including but not limited to blood, lymph, and CNS. Preferably, the peptides retain the other activities to result in an analog usable for vaccination or as an immunogen.

7. Decamers

[0171] The length of typical MHC binding peptides varies from about 8 to about 11 amino acids in length. However, most of the previously used HLA-A*0201 are 9-mers (nonamers) or 10-mers (decamers). Thus, in one embodiment, the analog can be a 10-mer of the wild-type sequence NY-ESO-$1_{157-166}$. However, because the wild-type 10-mer does not have the correct binding motif and showed no immunological activity, a 10-mer was created by substituting amino acids at the P10 position and identifying the effect of various wild-type and analogs (see Figures 13A-13C). In one embodiment, the residues that were added or substituted for the wild-type at the C-terminus can be selected from the group consisting of norvaline, leucine, isoleucine, valine, and alanine.

8. Remaining residues

[0172] With reference to Figures 13A and 13C, any residues can also be substituted with conservative amino acids. Conservative substitutions can be paired with any of the above substitutions that can produce an effect. Alternatively, conservative substitutions can be specifically at residues that are not believed to be involved in any of the activities at a primary, secondary, or even tertiary level. Such residues can include P3, P5 and/or P7. Conservative substitutions are known to those of skill in the art, but, for example, the Leucine at position P3 can be substituted with an alanine or threonine to produce an analog. Typically, such conservative substitutions do not significantly affect the activity of the analog. However, in some embodiments they may increase certain activities or decrease certain activities. Because of the known interactions, it is unlikely that such conservative substitutions will have a significant effect on any of the activities.

$PSMA_{288-297}$ Analogs

[0173] Many features regarding the variety of embodiments and aspects of analog design are disclosed above, either generally or as applied to particular epitopes. It is to be understood that such disclosure is also applicable to this and subsequent epitopes. Explicit restatement of such disclosure will be minimized for the sake of brevity.

[0174] Some embodiments relate to analogs of the MHC class I-restricted T cell epitope $PSMA_{288-297}$, GLPSIPVHPI, polypeptides comprising these analogs that can be processed by pAPC to present the epitope analogs, and nucleic acids that express the analogs. The analogs can have similar or improved immunological properties compared to the wild-type epitope. Evidence validating the presentation of this epitope by human cancer cells is presented in Example 32 below.

[0175] One embodiment relates to methods to derivatize and improve analogs of $PSMA_{288-297}$, along with specific sequences that encompass substitutions. The analogs can contain at least one substitution, but can have multiple substitutions comprising standard or non-standard amino

acids singly or in various combinations. The analogs may result in peptides with retained or improved properties.

[0176] Embodiments relate to families of one or more peptides of 9 or 10 amino acids in length related by sequence to amino acids 288-297 of the human PSMA.

Analog Design

[0177] In some embodiments, the $PSMA_{288-297}$ analog can contain substitutions of the sequence GLPSIPVHPI. Reference to binding motif data, such as presented in table 7 in example 2 below, indicates that the P2 anchor residue can make the largest individual contribution to affinity of any position in an A2.1-restricted epitope. In this case the amino acid at the P2 position is the optimally preferred leucine. The PΩ anchor residue, isoleucine, is favorable. *In vitro* binding studies using the T2 cell assay system (not shown) have indicated that the native peptide has generally superior binding characteristics, particularly as compared to the SSX-2 and NY-ESO-1 epitopes. The epitope exhibited significant binding at relatively low concentrations, although this was paired with a relatively shallow rise toward saturation. The wild-type epitope can be improved. Analyses such as that represented by tables 7 and 8 are averages and the behavior of a given residue in a particular sequence may diverge from the average. Consistent with the favorable results obtained with Nle and Nva for the SSX-2 and NY-ESO-1 epitopes discussed above, Nle and Nva also can be successfully used for the instant PSMA epitope. Finally, even similar binding characteristics, if paired with alterations that help circumvent whatever tolerance to the epitope may exist, can increase the effective immunogenicity of the peptide. In the transgenic mouse model the native peptide is poorly immunogenic (see Example 35 for instance) which may reflect tolerance to the epitope; the region of PSMA from which this epitope is derived is identical between mouse and human PSMA.

1. N-terminus proximal primary anchor modification (P2)

[0178] As noted above, although the native residue at the P2 position of this epitope is generally the optimal residue among genetically encoded amino acids, the effect of substituting other preferred or bulky hydrophobic residues were examined for potential improvement of binding, tolerance breaking and cross-reactive immunity. Exemplary substitutions can include Met, Ile, Gln, Val, Nva, Nle, and aminobutyric acid (Abu).

2. N-terminal secondary anchor modification (P1)

[0179] The N-terminal secondary anchor is the first amino acid at the N-terminus. The native Gly is only marginally preferred at this position. Various observations (see tables 7 and 8 for example) show that amino acids with potential to improve the epitope include Ala, Ser, Abu and sarkosine (Sar, that is, N-methylglycine).

3. C-terminal primary anchor modification (PΩ)

[0180] The native Ile at this position is generally a preferred but not optimal residue. Substitution at this position can improve binding. Exemplary substitutions can include Val, Leu, Nva, and Nle.

4. Secondary anchors and TCR exploration

[0181] The penultimate position (PΩ-1) can serve both as a secondary anchor and a TCR interacting position. Substitution of Ala, Leu, Ser, and Thr can be have their primary effect on TCR interaction, though they can also contribute to improved binding. P3 is another position that can effect both binding and immunogenicity. Substitution of Trp at this position can improve both.

[0182] Further embodiments relate to combinations of substitutions at multiple positions in order to combine, synergize, and counteract the various effects obtained with the single substitutions.

$PRAME_{425-433}$ Analogs**[0183]** Many features regarding the variety of embodiments and aspects of analog design are disclosed above, either generally or as applied to particular epitopes. It is to be understood that such disclosure is also applicable to this and subsequent epitopes. Explicit restatement of such disclosure will be minimized for the sake of brevity.

[0184] Embodiments include analogs of the MHC class I-restricted T cell epitope PRAME425-433, SLLQHLIGL, polypeptides comprising these analogs that can be processed by pAPC to present the epitope analogs, and nucleic acids that express the analogs. The analogs can have similar or improved immunological properties compared to the wild-type epitope. Evidence validating the presentation of this epitope by human cancer cells is presented in Example 39 below.

[0185] One embodiment relates to methods to derivatize and improve analogs of $PRAME_{425-433}$, along with specific sequences that encompass substitutions. The analogs can contain at least one substitution, but can have multiple substitutions comprising standard or non-standard amino acids singly or in various combinations. The analogs can result in peptides with retained or improved properties.

[0186] Some embodiments relate to families of one or more peptides of 9 or 10 amino acids in length related by sequence to amino acids 425-433 of the human PRAME sequence.

Analog Design

[0187] Some embodiments relate to analogs of the $PRAME_{425-433}$ which can contain substitutions of the sequence SLLQHLIGL. Reference to binding motif data, such as presented in table 7 in Example 2 below, indicates that the P2 anchor residue can make the largest individual contribution to affinity of any position in an A2.1-restricted epitope. In this case the amino acid at the

P2 position is the optimally preferred leucine. The PΩ anchor residue, leucine, is favorable, though not as strongly preferred. Analyses such as that represented by tables 7 and 8 are averages and the behavior of a given residue in a particular sequence can diverge from the average, nor is the wild type PΩ residue necessarilly the most preferred for that position. Consistent with the favorable results obtained with Nle and Nva for the other epitopes, similar improvements can be obtained substituting Nle and Nva with this sequence. Finally, even similar binding characteristics, if paired with alterations that help circumvent whatever tolerance to the epitope may exist, can increase the effective immunogenicity of the peptide.

[0188] The rationale for various substitutions has been set forth above. The particular substitutions investigated for the $PRAME_{425-433}$ epitope follow the same logic and are disclosed in the examples 40-42 and Figures 25-27. Substitutions were made at the primary anchor positions P2 and PΩ (P9), the secondary anchor positions P1 and PΩ-1 (P8). Substitutions were also made in the TCR interacting positions (in addition to secondary anchor positions) P3 and P6. Selected substitutions have impact on binding and/or stability of MHC class I – peptide complexes, key features in determining the immunological properties of peptides. In addition, due to T cell repertoire considerations and to circumvent mechanisms responsible for the limited immunity to native epitopes, substitutions that retain the capability of analogs to interact with T cell receptors recognizing native peptides, can be of practical value.

EXAMPLES

[0189] The following examples provide analogs and methods of identifying analogs. The analogs can be used, for example, as immunogens, vaccines, and/or treatment of a variety of cancers. The analogs were produced as in Example 1. $SSX\text{-}2_{41-49}$ analogs were identified as shown in Example 2, those produced listed in Example 3 and tested for improved properties as in Examples 4-21. The testing of NY-ESO-$1_{157-165}$ analogs were tested for improved properties as in Examples 22-30.

EXAMPLE 1

Peptide synthesis, purification and characterization

[0190] Peptides were synthesized on either a Symphony multiple peptide synthesizer (PTI technologies, MA) or an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) at 0.05 – 0.1mmole scale using standard Fmoc solid phase chemistry. C-terminal free acid peptides were synthesized using pre-load PEG-PS resins (on Symphony) or Wang resin (on ABI). C-terminal amidated peptides were synthesized on Fmoc-PAL-PEG-PS resin. All resins were purchased from Applied Biosystems (Foster City, CA). The Fmoc-amino acids used in peptide syntheses were purchased from Novabiochem (San Diego, CA) and AnaSpec (San Jose, CA). Post-synthesis cleavage was carried on by the standard protocol.

[0191] Peptide purification was carried out on either semi-preparative HPLC columns or SPE cartridges (Phenomenex, Torrance, CA). The purity of all peptides was ≥ 90%. The identity of each peptide was verified by Maldi-TOF MS (Voyager DE, Applied Biosystems) and analytical HPLCs (Varian or Shimazu) using a Synergi C12 column (Phenomenex, Torrance, CA).

EXAMPLE 2

De Novo Designed SSX-241-49 Analogs

[0192] Structural modification of a moderately antigenic peptide can considerably improve peptide-MHC binding, CTL recognition, and/or immunogenicity. General guidelines regarding how to modify a wild-type epitope in order to achieve a peptide analog with enhanced potency are known in the art. An appreciated strategy is to optimize the residues at the so-called anchor positions for binding to the particular MHC molecule at issue. In the case of HLA-A2 a marked preference for hydrophobic residues at the P2 and PΩ positions has been observed, particularly L, and M at P2, and V at PΩ. (PΩ denotes the C-terminal residue of the epitope. For HLA-A2 that is P9 or P10 depending on the length of the peptide.) Replacing the P1 position with aromatic residues, such as F, Y and W can also be advantageous.

Table 7. Coefficients used by the BIMAS algorithm

(Algorithm available by hypertext transfer protocol: //bimas.cit.nih.gov/molbio/hla_bind/)

9-mer Coefficient Table for HLA_A_0201									
Amino Acid Type	Position								
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	1.000	0.470	1.000	1.000	1.000	1.000	1.000	1.000	1.000
D	0.075	0.100	0.400	4.100	1.000	1.000	0.490	1.000	0.003
E	0.075	1.400	0.064	4.100	1.000	1.000	0.490	1.000	0.003
F	4.600	0.050	3.700	1.000	3.800	1.900	5.800	5.500	0.015
G	1.000	0.470	1.000	1.000	1.000	1.000	0.130	1.000	0.015
H	0.034	0.050	1.000	1.000	1.000	1.000	1.000	1.000	0.015
I	1.700	9.900	1.000	1.000	1.000	2.300	1.000	0.410	2.100
K	3.500	0.100	0.035	1.000	1.000	1.000	1.000	1.000	0.003
L	1.700	72.000	3.700	1.000	1.000	2.300	1.000	1.000	4.300
M	1.700	52.000	3.700	1.000	1.000	2.300	1.000	1.000	1.000
N	1.000	0.470	1.000	1.000	1.000	1.000	1.000	1.000	0.015
P	0.022	0.470	1.000	1.000	1.000	1.000	1.000	1.000	0.003
Q	1.000	7.300	1.000	1.000	1.000	1.000	1.000	1.000	0.003
R	1.000	0.010	0.076	1.000	1.000	1.000	0.200	1.000	0.003
S	1.000	0.470	1.000	1.000	1.000	1.000	1.000	1.000	0.015

9-mer Coefficient Table for HLA_A_0201									
Amino Acid Type	**Position**								
	1st	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**8th**	**9th**
T	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.500
V	1.700	6.300	1.000	1.000	1.000	2.300	1.000	0.410	14.000
W	4.600	0.010	8.300	1.000	1.000	1.700	7.500	5.500	0.015
Y	4.600	0.010	3.200	1.000	1.000	1.500	1.000	5.500	0.015
final constant	0.069								

Table 8. Scoring Pattern for HLA-A*0201 used by the SYFPEITHI Algorithm (9-mers)

(Algorithm available by hypertext transfer protocol: //syfpeithi.bmi-heidelberg.com/scripts/MHCServer.dll/home.htm)

AA	P1	P2	P3	P4	P5	P6	P7	P8	P9
A	2	6	2	0	0	0	2	1	6
C	0	0	0	0	0	0	0	0	0
D	-1	0	0	1	0	0	0	0	0
E	-1	0	-1	2	0	0	0	2	0
F	1	0	1	-1	1	0	0	0	0
G	1	0	0	2	2	0	0	1	0
H	0	0	0	0	0	0	1	0	0
I	2	8	2	0	0	6	0	0	8
K	1	0	-1	0	1	0	-1	2	0
L	2	10	2	0	1	6	1	0	10
M	0	8	1	0	0	0	0	0	6
N	0	0	1	0	0	0	1	0	0
P	0	0	0	2	1	0	1	0	0
Q	0	0	0	0	0	0	0	0	0
R	0	0	0	0	0	0	0	0	0
S	2	0	0	0	0	0	0	2	0
T	0	6	-1	0	0	2	0	2	6
V	1	6	0	0	0	6	2	0	10
W	0	0	1	0	0	0	0	0	0
X	0	0	0	0	0	0	0	0	0
Y	2	0	1	-1	1	0	1	0	0

[0193] Adapted from: Rammensee, Bachmann, Stevanovic: *MHC ligands and peptide motifs*. Landes Bioscience 1997

EXAMPLE 3

The following analogs were produced using the predictions in Example 1.

Table 9.

Catergory	SEQ ID Number	Peptide name	Sequence
wild-type	1	**SSX-2 41-49**	KASEKIFYV
N-terminal Primary Anchor	2	SSX-2 41-49 (A42L)	**K**LSEKIFYV
	3	SSX-2 41-49 (A42V)	**K**VSEKIFYV
	4	SSX-2 41-49 (A42I)	**K**ISEKIFYV
	5	SSX-2 41-49 (A42M)	**K**MSEKIFYV
	6	SSX-2 41-49 (A42(D-Ala))	**K(D-Ala)**SEKIFYV
	7	SSX-2 41-49 (A42(D-Leu))	**K(D-Leu)**SEKIFYV
	8	SSX-2 41-49 (A42(D-Val))	**K(D-Val)**SEKIFYV
	9	SSX-2 41-49 (A42(Nal-1))	**KNal-1**SEKIFYV
	10	SSX-2 41-49 (A42(Nal-2))	**KNal-2**SEKIFYV
	11	SSX-2 41-49 (A42(Abu))	**KAbu**SEKIFYV
	12	SSX-2 41-49 (A42(Nle))	**KNle**SEKIFYV
	13	SSX-2 41-49 (A42(Nva))	**KNva**SEKIFYV
	14	SSX-2 41-49 (A42(Aib))	**KAib**SEKIFYV
N-terminal Secondary Anchor	15	SSX-2 41-49 (K41F)	**F**ASEKIFYV
	16	SSX-2 41-49 (K41W)	**W**ASEKIFYV
	17	SSX-2 41-49 (K41Y)	**Y**ASEKIFYV
	18	SSX-2 41-49(K41(D-Lys))	**(D-Lys)**ASEKIFYV
	19	SSX-2 41-49 (K41(Phg))	**Phg**ASEKIFYV
	20	SSX-2 41-49 (K41(Cha))	**Cha**ASEKIFYV
	21	SSX-2 41-49 (K41(Phe-4F))	**Phe(4-F)**ASEKIFYV
	22	SSX-2 41-49 (K41(Phe-4NO2))	**Phe(4-NO_2)**ASEKIFYV
	23	SSX-2 41-49 (K41(O-methyl Tyr))	**O-methyl-Tyr**ASEKIFYV
	24	SSX-2 41-49 (K41(□-(3-benzothienyl)Ala))	**□-(3-benzothienyl)Ala**ASEKIFYV
N-terminal Primary/Secondary Anchor	25	SSX-2 41-49 (K41Y, A42L)	**YL**SEKIFYV
	26	SSX-2 41-49 (K41Y, A42V)	**YV**SEKIFYV
	27	SSX-2 41-49 (K41Y, A42M)	**YM**SEKIFYV
	28	SSX-2 41-49 (K41Y, A42I)	**YI**SEKIFYV
	29	SSX-2 41-49 (K41F, A42L)	**FL**SEKIFYV
	30	SSX-2 41-49 (K41F, A42V)	**FV**SEKIFYV
	31	SSX-2 41-49 (K41F, A42M)	**FM**SEKIFYV
	32	SSX-2 41-49 (K41F, A42I)	**FI**SEKIFYV
	33	SSX-2 41-49 (K41W, A42L)	**WL**SEKIFYV
	34	SSX-2 41-49 (K41W, A42V)	**WV**SEKIFYV
	35	SSX-2 41-49 (K41W, A42M)	**WM**SEKIFYV
	36	SSX-2 41-49 (K41W, A42I)	**WI**SEKIFYV
	37	SSX-2 41-49 (K41(D-Lys), A42L)	**(D-Lys)L**SEKIFYV
	38	SSX-2 41-49 (K41(D-Lys), A42V)	**(D-Lys)V**SEKIFYV

Catergory	SEQ ID Number	Peptide name	Sequence
N-terminal Primary/Secondary Anchor, C-terminal Primary Anchor	39	SSX-2 41-49 (K41F, A42V, V49L)	**FVSEKIFYL**
	40	SSX-2 41-49 (K41F, A42V, V49I)	**FVSEKIFYI**
	41	SSX-2 41-49 (K41F, A42V, V49A)	**FVSEKIFYA**
	42	SSX-2 41-49 (K41F, A42V, V49M)	**FVSEKIFYM**
	43	SSX-2 41-49 (K41F, A42V, V49Nle)	**FVSEKIFY(Nle)**
	44	SSX-2 41-49 (K41F, A42V, V49Nva)	**FVSEKIFY(Nva)**
	45	SSX-2 41-49 (K41F, A42V, V49MeVal)	**FVSEKIFY(MeVal)**
	46	SSX-2 41-49 (K41F, A42V, V49MeLeu)	**FVSEKIFY(MeLeu)**
	47	SSX-2 41-49 (K41F, A42V, V49Aib)	**FVSEKIFY(Aib)**
	48	SSX-2 41-49 (K41F, A42V, V49Abu)	**FVSEKIFY(Abu)**
N-terminal Primary/Secondary Anchor, TCR sites	49	SSX-2 41-49 (K41F, A42V, E44D)	**FVSDKIFYV**
	50	SSX-2 41-49 (K41F, A42V, E44N)	**FVSNKIFYV**
	51	SSX-2 41-49 (K41F, A42V, E44S)	**FVSSKIFYV**
	52	SSX-2 41-49 (K41F, A42V, E44T)	**FVSTKIFYV**
	53	SSX-2 41-49 (K41F, A42V, E44Q)	**FVSQKIFYV**
	54	SSX-2 41-49 (K41F, A42V, E44(Nle))	**FVS(Nle)KIFYV**
	55	SSX-2 41-49 (K41F, A42V, E44(Nva))	**FVS(Nva)KIFYV**
	56	SSX-2 41-49 (K41F, A42V, I46L)	**FVSEKLFYV**
	57	SSX-2 41-49 (K41F, A42V, I46V)	**FVSEKVFYV**
	58	SSX-2 41-49 (K41F, A42V, I46M)	**FVSEKMFYV**
	59	SSX-2 41-49 (K41F, A42V, I46(Nle))	**FVSEK(Nle)FYV**
	60	SSX-2 41-49 (K41F, A42V, I46(Nva))	**FVSEK(Nva)FYV**
	61	SSX-2 41-49 (K41F, A42V, Y48T)	**FVSEKIFTV**
	62	SSX-2 41-49 (K41F, A42V, Y48F)	**FVSEKIFFV**
	63	SSX-2 41-49 (K41F, A42V, Y48S)	**FVSEKIFSV**
	64	SSX-2 41-49 (K41F, A42V, Y48(Phe-4F))	**FVSEKIF(Phe4-F)V**
	65	SSX-2 41-49 (K41F, A42V, Y48(Phg))	**FVSEKIF(Phg)V**
	66	SSX-2 41-49 (K41F, A42V, I46L,Y48T)	**FVSEKLFTV**
	67	SSX-2 41-49 (K41F, A42V, I46L,Y48S)	**FVSEKLFSV**
N-terminal Primary/Secondary Anchor, C-terminal Primary Anchor, TCR sites	68	SSX-2 41-49 (K41F, A42V, I46L,Y48T, V49A)	**FVSEKLFTA**
	69	SSX-2 41-49 (K41F, A42V, I46L,Y48S, V49A)	**FVSEKLFSA**
N-terminal Primary Anchor, C-terminal Primary Anchor	70	**SSX-2 41-49 (A42V, V49I)**	**KVSEKIFYI**
	71	SSX-2 41-49 (A42L, V49I)	**KLSEKIFYI**
	72	SSX-2 41-49 (A42(D-Ala), V49(D-Val))	**K(D-Ala)SEKIFY(D-Val)**
	73	SSX-2 41-49 (A42(D-Leu), V49(D-Val))	**K(D-Leu)SEKIFY(D-Val)**
	74	SSX-2 41-49 (A42(D-Val), V49(D-Val))	**K(D-Val)SEKIFY(D-Val)**
C-terminal Primary Anchor	75	SSX-2 41-49 (V49I)	**KASEKIFYI**
C-terminal amide	76	SSX-2 41-49-NH2	KASEKIFYV-NH2

Catergory	SEQ ID Number	Peptide name	Sequence
	77	SSX-2 41-49-NH2 (A42L)	KLSEKIFYV-NH2
	78	SSX-2 41-49-NH2 (A42V)	KVSEKIFYV-NH2
Decamers	79	SSX-2 41-50	KASEKIFYVY
	80	SSX-2 41-50 (Y50I)	KASEKIFYVI
	81	SSX-2 41-50 (Y50L)	KASEKIFYVL
	82	SSX-2 41-50 (Y50V)	KASEKIFYVV
	83	SSX-2 41-50 (Y50 (Nle))	KASEKIFYV(Nle)
	84	SSX-2 41-50 (Y50 (Nva))	KASEKIFYV(Nva)
	85	SSX-2 41-50 (A42V,Y50I)	KVSEKIFYVI
	86	SSX-2 41-50 (A42L,Y50I)	KLSEKIFYVI
	87	SSX-2 41-50 (A42V,Y50L)	KVSEKIFYVL
	88	SSX-2 41-50 (A42L,Y50L)	KLSEKIFYVL
	89	SSX-2 41-50 (A42V,Y50V)	KVSEKIFYVV
	90	SSX-2 41-50 (A42L,Y50V)	KLSEKIFYVV
	91	SSX-2 41-50 (A42V,Y50(Nle))	KVSEKIFYV(Nle)
	92	SSX-2 41-50 (A42L,Y50(Nle))	KLSEKIFYV(Nle)
	93	SSX-2 41-50 (A42V,Y50(Nva))	KVSEKIFYV(Nva)
	94	SSX-2 41-50 (A42L,Y50(Nva))	KLSEKIFYV(Nva)
	95	SSX-2 41-50 (A42V,V49I,Y50I)	KVSEKIFYII
	96	SSX-2 41-50 (A42L,V49I,Y50I)	KLSEKIFYII
	97	SSX-2 41-50 (V49I,Y50I)	KASEKIFYII

[0194] Abbreviations for non-standard amino acids: Nle, norleucine; Nva, norvaline; Phg, phenylglycine; Phe(4-F), 4-fluorophenylalanine; Phe(4-NO_2), 4-nitrophenylalanine; Abu, α-aminobutyric acid; Aib, α-aminoisobutyric acid; MeLeu, methyl-leucine; MeVal, methylvaline; β-(3-benzothienyl)Ala, β-(3-benzothienyl)-alanine; O-methyl-Tyr, O-methyltyorosine; Cha, cyclohexylalanine; Nal-1, β-(1-napthyl)-alanine; Nal-2, β-(2-napthyl)-alanine; -NH2 indicates that the carboxy terminus has been modified to the amide.

EXAMPLES 4-21

TESTING OF SSX-2_{41-49} ANALOGS

[0195] The analogs produced in Example 3 were tested for activity, such as binding and biological effect as follows in Examples 4-21:

EXAMPLE 4

Peptide Binding Using T2 Cells

[0196] The affinity of peptide analogs and the wild-type epitope to HLA-A*0201 was evaluated using a T2 cell based assay (Regner M, et al., *Exp Clin Immunogenet.* 1996;13(1):30-5; which is hereby incorporated by reference in its entirety).

[0197] For the binding assay, in brief, the T2 cells that lack expression of TAP and thus do not assemble stable MHC class I on the cell surface, were pulsed with different concentrations of peptides (controls or analogs) overnight at 37°C, washed extensively, stained with fluorescently tagged antibody recognizing MHC class I (A2 allele) and run through a FacsScan analyzer. The

difference between the MFI (mean fluorescence intensity) corresponding to a given concentration of analog and the negative control (non-MHC binder) is a function of how many stabilized complexes between MHC and peptide are displayed on the surface of T2 cells. Thus, at limiting concentrations of peptide, this is a measurement of K_{on} mostly and at saturation levels of peptide that is a measurement of both K_{on} and K_{off}. The binding was quantified by two factors that are mathematically related: half maximal binding (the peptide concentration giving 50% of the signal corresponding to saturation) and relative affinity (1/RA). Relative affinity RA is binding normalized to a reference (wild-type peptide); for example, the ratio between half max binding of control relative to peptide analog. The higher the 1/RA index and the lower the half maximal binding, the higher the K_{on} of the interaction between the analog and the MHC. Fifty three analogs were identified with these binding parameters improved relative to the wild-type peptide. These improved binders carry one, two, three or multiple substitutions (including standard and/or non-standard amino acids) involving positions that are known to participate in the interaction with MHC and/or TCR. However, the overall effect on MHC binding was dependent on the modification. Such peptide analogs can be useful in therapeutic compositions or as a platform to further derive therapeutic compositions.

EXAMPLE 5

PEPTIDE STABILITY USING T2 CELLS

[0198] Peptide stability (K_{off}) on MHC generally cannot be solely inferred from binding (K_{on}). In addition, along with binding, the stability of peptides on MHC class I is notoriously important in regard to the immunological properties of such peptides, since the activation of T cells depends on the duration of "signal 1" (MHC peptide complex interaction with T cell receptor). For the stability assay, in brief, the T2 cells that lack expression of TAP and thus do not assemble stable MHC class I on the cell surface, were pulsed with a concentration of peptide (controls or analogs) known to achieve maximal loading of MHC class I ("saturation") overnight at 37°C, washed extensively, and chased for different intervals in the presence of emetine, which blocks endogenous protein synthesis. After extensive washing, the cells were stained with fluorescently tagged antibody recognizing MHC class I (A2 allele) and run through a FacsScan analyzer. The difference between the MFI (mean fluorescence intensity) corresponding to a given concentration of analog and the negative control (non-MHC binder) is a function of how many stabilized complexes between MHC and peptide are displayed on the surface of T2 cells. The decay of the signal over time was mathematically expressed as stability index 50% relative to the binding at 0 hours (at the beginning of the chase interval).

[0199] Such improved analogs can carry one, two, three or multiple substitutions (including standard and/or non-standard amino acids) involving positions that are known to participate in the interaction with MHC and/or TCR, with an overall effect on MHC stability that is dependent on the modification. Such peptide analogs can be useful in therapeutic compositions or as

a platform to further derive therapeutic compositions. Forty three of the analogs have increased stability relative to the natural peptide.

[0200] The analogs that showed both improved binding and stability are useful in improved compositions or as a platform to generate improved compositions of therapeutic benefit.

Example 6

EVALUATION OF IMMUNOLOGIC PROPERTIES OF ANALOGS:
CROSS-REACTIVITY AND FUNCTIONAL AVIDITY

[0201] The immunologic properties of peptides can be described as a function of binding to MHC molecules (K_{on} and K_{off}) and TCR (affinity of interaction between TCR and MHC-peptide complexes). Modifications of primary MHC anchor residues generally have a significant degree of predictability in regard to overall impact on binding to MHC molecules.

[0202] Modifications of secondary MHC anchor residues may impact the affinity of interaction of the MHC-peptide complex to TCR along with the K_{on} and K_{off} relative to peptide-MHC interaction.

[0203] A methodology was devised that allowed rapid and rational screening of peptide analogs in a fashion coherent with proposed methods of use and modeling the overall immunologic properties (K_{on} and K_{off} relative to MHC interaction and TCR binding properties in an integrated fashion). This method can include generating T cell lines against a natural (non-mutated) epitope ($SSX\text{-}2_{41\text{-}49}$) using an immunization strategy potent enough to generate a useful response in transgenic mice carrying human MHC (such as the A2 allele). Peptide analogs were interrogated *ex vivo* in the presence of competent APCs and the functional impact of T cells specific for natural (non-mutated) epitope measured. The evaluation was done at various concentrations of analog, since the expected effect was biphasic in the case of cross reactive peptides (activating at limited concentrations and inhibiting at higher concentrations, due to antigen-induced cell death, AICD). Measurement of the following three parameters can define basic and useful characteristics of peptide analogs:

1. Minimal required concentration of peptide analog to trigger effects indicative of T cell activation (e.g. cytokine production);
2. Maximal (peak value) effect (e.g. cytokine production) at any analog concentration;
3. Analog concentration at peak value of activating effect (*e.g.*, cytokine concentration)

[0204] For example, analogs that result in reduced values associated with parameters #1 and 3 but increased #2, can be useful. Use of natural epitope and unrelated non-cross reactive peptides as references is valuable in identifying classes of analogs of potential value. Analogs that display properties quantitatively comparable to or even modestly attenuated from those of natural epitopes are still deemed useful in light of the fact that while they retain cross-reactivity, they may

display immunologic properties that are distinct from those of the natural peptide – for example lower propensity to induce AICD or ability to break tolerance or restore responsiveness *in vivo*.

[0205] Some advantages of this screening strategy include the practicality and rapidity, use of more relevant polyclonal T cell lines instead of potentially biased T cell clones as a read out, and the composite value, integrating parameters such as K_{on}, K_{off} and TCR affinity that may translate into cross-reactivity and functional avidity of peptide-MHC complexes relative to TCR. These parameters can be predictive of the *in vivo* immunologic properties and thus can delineate useful panels of peptide analogs to undergo further evaluation, optimization and practical applications. Analogs that bind to MHC and retain cross-reactivity against TCR specific for the nominal wild-type peptide are predicted to trigger a measurable effect in this assay. The overall methodology is presented in Figure 2.

[0206] The method used for the generation of T cell lines was the following: HHD transgenic mice carrying an A2 human allele (Pascolo et al. J. Exp Med. 185(12):2043-51, 1997, which is hereby incorporated herein by reference in its entirety) were immunized with 50ug of SSX-2 natural epitope (41-49) admixed with 25ug of pIpC at day 0, 4, 14 and 18 by bilateral administration into the inguinal lymph nodes. At 7 days after the last boost, the mice were sacrificed and a suspension of splenocytes prepared at $5x10^6$ million cells/ml in complete HL-1 medium. Cells were incubated with different concentrations of peptide for 48 hours in flat-bottomed 96-well plates (200ul/well) and for an additional 24 hours with rIL-2 at 10U/ml added to the wells. The supernatant was harvested and the concentration of IFN-gamma assessed by standard methods such as ELISA.

EXAMPLE 7

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT SINGLE POSITION

[0207] The strategy from above (Example 6, Figure 2) was applied to scan through a library of analogs bearing single substitutions relative to the natural SSX-2_{41-49} epitope (KASEKIFYV) in its wild-type version (Figure 3). Strong inverse correlation was found between the minimal required amount of analog to elicit IFN-gamma production *ex vivo* and the maximal amount of cytokine production at any concentration of analog.

[0208] Substitution of A_{42} with L, V or M improved on the immunologic properties of the peptide, assessed in this assay. L and V mutants were active. M was more active than the natural epitope. The I mutant retained cross-reactivity to the TCR recognizing the wild-type epitope.

[0209] Replacement of the A at position 42 with non-standard amino acids Abu, Nle or Nva improved on the immunologic properties of the peptide relative to the wild-type epitope, both in terms of the minimal amount of analog required to trigger cytokine production and the peak amount of cytokine produced. Mutants encompassing D-Ala, D-Val, Nal-2 or Aib display retained cross-reactivity and reduced immune activity in this assay relative to the natural peptide, but can still be

useful for further derivitization to adjust or enhance their properties. An Nal-1 at position 42 abrogated the activity.

[0210] Changes of the first residue K_{41} showed that, while replacement with F or Phg improved on the activity, W, D-Lys, and Cha obliterated the immunologic properties in this assay. Replacement of K with Y, Phe-4F, Phe(4-NO2), O-methyl-Tyr or beta-(3-benzothienyl-Ala) retained activity.

[0211] Modification of position V_{49} (C-terminal residue) by replacement with I retained the activity at a lower level compared to the original epitope. Modification of the last residue by addition of an –NH2 moiety obliterated the activity of the peptide that was subsequently rescued by modifying the A at position 42 with L or V. This illustrates directly that analogs with activity that is lower than that of the wild-type peptide are still useful for further derivatization.

EXAMPLE 8

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT TWO POSITIONS

[0212] The strategy from above (Example 6, Figure 2) has been applied to scan through a library of analogs bearing two substitutions, relative to the wild-type SSX-2_{41-49} epitope in its wild-type version (Figure 4).

[0213] Coordinated modifications at position 1 and 2 have a variable effect on the activity of analogs. For example, substitution of K41 with Y, F or W corroborated with substitution of A42 with V, M or I, and resulted in preserved or enhanced activity of the analogs relative to the wild-type peptide. Such doubly mutated peptides offer an increased opportunity to impact the interaction with TCR in a fashion that results in tolerance breaking (thus being useful for practical application), since the P1 residue participates to a certain extent in binding to TCR. Combinations between the following: Y (position 41) with V (at position 42), W (position 41) with I or I (at position 42), and F (position 41) with L, V, I (at position 42) resulted in analogs that were more active relative to the wild-type peptide. Combinations between Y at position 41 and I at position 42, or W at position 41 and V or M at 42, conferred an activity similar to that of wild-type peptide. Replacement of K with D-lysine at position 41 reduced resulted in analogs with retained activity in this assay. Such peptides can be very useful since the metabolic degradation of such peptides encompassing non-standard amino acids is decreased *in vivo*.

[0214] Combinations between V or L at position 42 and I at position 49 resulted in increased activity over the natural peptide.

EXAMPLE 9

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT MULTIPLE POSITIONS

[0215] The strategy from above (Example 6, Figure 2) has been applied to scan through a library of analogs bearing three or more substitutions relative to the natural SSX-2_{41-49} epitope in its wild-type version (Figure 5).

[0216] F and V at positions 41 and 42 respectively, combined with I or A at position 49 resulted in improved or similar activity relative to the wild-type epitope. In contrast, L or M at position 49 resulted in heavily diminished activity.

[0217] Triple mutants comprising the non-standard amino acids Nva, Abu or MeVal at the last position resulted in retention or improvement of immune activity. Such peptides are extremely useful due to increased *in vivo* stability and resistance to enzymatic degradation.

[0218] Modification of amino acid residues within the putative TCR binding region can result in peptides of considerable value, that retain binding to MHC along with cross-reactivity and thus be useful for restoration of immune responsiveness or tolerance breaking since their conformation in the MHC groove is slightly different from that of natural peptides. Additional substitutions at position 44 (Q, Nva or Nle), position 46 (L, V, Nle or Nva) or 48 (F or Phe-4F) resulted in active analogs, whereas D, N, S or T at position 44, M at 46 or T, S, Phg at position 48 or L at position 46 with T at 48, resulted in analogs devoid of activity. Finally, two analogs with 5 substitutions showed no activity (Figure 5).

EXAMPLE 10

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF DECAMERS ENCOMPASSING THE NATURAL PEPTIDE AND MUTATED AT VARIOUS POSITIONS

[0219] The strategy from above (Example 6, Figure 2) has been applied to scan through a library of analogs of a decamer encompassing the nominal SSX-2_{41-49} peptide (Figure 6).

[0220] The decamer SSX-2 41-50 was significantly less active in stimulating the T cell line specific for the 41-49 nonamer, relative to the latter. Modification of the Y residue at position 50 to I or L, but to a lesser or no extent to V, Nle or Nva, resulted in restoration of activity in this assay. Further optimization of the activity of decameric analogs can be obtained by modification of the A at position 2 with L or V. The A42L substitution rescued the activity of the Y50Nva decamer. Peptide analogs of similar or reduced activity *in vitro* (but retained cross-reactivity) compared with the natural peptide are still useful for induction or boost of immune responses due to: i) more limited AICD; ii) potentially higher *in vivo* activity due to increased stability on class I MHC and/or slightly modified interaction with TCR which is can be important for tolerance breaking.

EXAMPLE 11

USE OF ANALOGS TO TRIGGER ENHANCED IMMUNITY AGAINST NATURAL EPITOPE, ASSESSED *EX VIVO*

[0221] Three groups of mice (n=4) were immunized with a plasmid expressing SSX-2_{41-49} natural epitope, by direct inoculation into the inguinal lymph nodes with 25ug in 25ul of PBS/each lymph node at day 0, 3, 14 and 17. This was followed by two additional peptide boosts (similar amount) at day 28 and 31. The schedule of immunization is shown in Figure 7. One week after the boost, splenocytes were stimulated *ex vivo* with SSX-2_{41-49} natural peptide and tested against ^{51}Cr-labeled target cells (T2 cells) at various E:T ratios (Figure 8). The results showed that the analog A42V triggered a higher response against target cells expressing the natural peptide, compared to the analog A42L or the wild-type peptide itself, as boost agents. This correlated with the binding and stability parameters determined by *ex vivo* experimentation.

EXAMPLE 12

USE OF ANALOGS TO TRIGGER ENHANCED IMMUNITY AGAINST NATURAL EPITOPE, ASSESSED *IN VIVO*

[0222] Eight groups of mice (n=4) were immunized with plasmid expressing SSX-2_{41-49} natural epitope, by direct inoculation into the inguinal lymph nodes with 25ug in 25ul of PBS/each lymph node at day 0, 3, 14 and 17. This was followed by two additional peptide boosts (similar amount) at day 28 and 31, using a negative control peptide (Melan A 26-35 "EAA"), natural peptide or analogs as shown in Figure 9.

[0223] To evaluate the *in vivo* response against natural peptide, splenocytes were isolated from littermate control HHD mice and incubated with 20μg/mL or 1ug/ml of natural peptide for 2 hours. These cells were then stained with $CFSE^{hi}$ fluorescence (4.0μM or 1μM for 15 minutes) and intravenously co-injected into immunized mice with an equal number of control splenocytes stained with $CFSE^{lo}$ fluorescence (0.4μM). Eighteen hours later the specific elimination of target cells was measured by removing spleen and PBMC from challenged animals and measuring CFSE fluorescence by flow cytometry. The relative depletion of the populations corresponding to peptide loaded splenocytes was calculated relative to the control (unloaded) population and expressed as % specific lysis. Figure 10 (spleen) and 11 (blood) show the *in vivo* cytotoxicity elicited by the regimens described in Figure 7. Three of the tested peptides (A42V, K41F and K41Y) showed increased activity relative to the natural peptide, both in spleen and blood, against target cells coated with 20 as well as 1ug/ml of natural peptide. Interestingly, there was only limited correlation between binding, stability of analogs in regard to the interaction with MHC, and the capability to generate *in vivo* immunity against natural peptide (Figure 11).

EXAMPLE 13

USE OF ANALOGS TO TRIGGER ENHANCED RESPONSES AGAINST TUMOR CELLS

[0224] Eight groups of mice (n=4) were immunized with plasmid expressing SSX-2_{41-49} natural epitope, by direct inoculation into the inguinal lymph nodes with 25ug in 25ul of PBS/each lymph node at day 0, 3, 14 and 17. This was followed by two additional peptide boosts (similar amount) at day 28 and 31, using a negative control peptide (Melan A 26-35 "EAA"), natural peptide or analogs as shown in Figure 9.

[0225] One week after the boost, splenocytes were stimulated *ex vivo* with SSX-2_{41-49} wild-type peptide and tested against ^{51}Cr-labeled human tumor cells (624.38 melanoma cells) at various E:T ratios (Figure 12). Analog A42V and K41F A42V V49I elicited immune responses that mediated increased cytotoxicity against human tumor cells expressing the natural SSX-2_{41-49} epitope.

EXAMPLE 14

N-TERMINAL PROXIMAL PRIMARY ANCHOR MODIFICATION(2^{ND} AA)

[0226] When the substituted analogs shown in Table 3 were tested, the analogs showed improved binding and stability profiles in comparison with the wild-type peptide epitope. However, the magnitude of improvement for each analog varied, and the substitution of A42V showed the highest improvement in terms of binding affinity with HLA-A*0201 molecule. Further, the stability of the A42V-HLA-A*0201 complex was better than the complex formed between wild-type peptide and HLA-A*0201: the T1/2 extended from 11.5 hrs to 20 hrs. The peptides with 42 A to L, V and M substitutions were able to induce the IFN-γ secretion of wild-type peptide specific CTL at remarkable lower concentrations. The 42A to I substitution generated an analog with improved binding and stability profile. The residue at the P2 position can also be engaged in the interaction with TCR to a certain degree. This observation was also supported by the results with the 42 A to Aib analog, which possessed a similar binding affinity with HLA-A*0201 relative to the wild-type epitope.

EXAMPLE 15

N-TERMINAL SECONDARY ANCHOR MODIFICATION (1^{ST} AA)

[0227] The N-terminal secondary anchor is the first amino acid at the N-terminus. Thus, in one embodiment, the original Lys 43 found in the wild-type sequence is substituted with a more hydrophobic and bulky amino acid. Any more hydrophobic and bulky amino acid also can be used, including any available to or that is known to one of skill in the art, including standard amino acids and non-standard amino acids. Examples of more hydrophobic amino acids include, but are not limited to: Phe, Tyr, Trp, and D-Lys.

[0228] The residue of Lys 41 was defined as a secondary anchor residue in interacting with HLA-A*0201 molecule, and it also engaged in the interaction with the T cell receptors to a

certain degree. Therefore, modifications of this position can generate some heteroclitic analogs that are more immunogenic and more suitable for the development of tumor vaccines.

[0229] From Table 3, one could see that by replacing Lys 41 to Tyr, Phe or Phe derivatives (Phenylglycine, Para-fluorophenylalanine, Para-nitrophenylalanine), the resulting analogs have higher affinity with the HLA-A*0201 molecule and form more stable complexes. On the other hand, the Lys to Trp or Trp derivatives analogs have shown significantly decreased affinity with the HLA-A*0201 molecule although based on the predicted algorithms, the Trp analog should have a similar affinity to that of the Tyr and Phe analogs. The experimental data have demonstrated the limitation of the predicted algorithms. For examples: Lys 41 to Phg substitution has resulted in an analog with improved affinity and extended stability with the HLA-A*0201 molecule, however, its cross-reactivity with wild-type peptide specific CTL was fairly poor. On the other hand, the para-nitrophenylalanine analog was shown to induce the IFN-γ secretion of the wild-type peptide specific CTL at a much lower concentration, although its affinity with the HLA-A*0201 molecule was about the same as that of wild-type peptide.

EXAMPLE 16

N-TERMINAL PRIMARY/SECONDARY ANCHOR MODIFICATION

[0230] When both primary and secondary anchor residues at the N-terminal were modified, a general trend was that resulting analogs demonstrated improved affinity and extended stability with the HLA-A*0201 molecule (Table 3), with only a few exceptions: (K41Y, A42V), (K41Y, A42M) and (K41(D-Lys), A42V). Additionally, they had very good cross-reactivity with the wild-type peptide specific CTL. Combining the K41W substitution with A42V or A42L improved the binding/stability profile, these analogs and also had desirable cross-reactivity activity with the wild-type peptide. The combination modifications of N-terminal primary anchor and secondary anchor changed the peptide structure and conformation to a greater degree.

EXAMPLE 17

N-TERMINAL PRIMARY/SECONDARY ANCHOR AND C-TERMINAL PRIMARY MODIFICATION

[0231] The C-terminal Val of the wild-type peptide was a preferred anchor residue. However, improved potency was observed when it was mutated to Ile, having one additional –CH2 group; similar improvement was also observed with a Val to Abu substitution. Although the other analogs showed improved binding affinity and stability with the MHC molecule, their cross-reactivity results were poor. The results of these analogs indicated that the peptide C-terminal anchor residue also plays a critical role in the recognition of T cells. (Table 4).

EXAMPLE 18

N-TERMINAL PRIMARY/SECONDARY ANCHOR AND TCR SITES MODIFICATION

[0232] Substitutions of secondary TCR binding amino acid residues preferably generate heteroclitic analogs that did not interfere with the binding to the MHC molecule, but overcame the tolerance issues of self-antigens. By combining the substitutions of N-terminal primary/secondary anchor residues (K41F and A42V) and the TCR sites, analogs were generated with improved binding affinity and stability (Table 6). Some of these analogs induced the IFN-γ production of the wild-type peptide specific CTL at lower concentrations, such as K41F, A42V, E44(Nva) /(Nle) mutants and K41F, A42V, I46L/(Nva) /(Nle) mutants.

EXAMPLE 19

N-TERMINAL AMIDE

[0233] Replacing the peptide's free carboxylic acid C-terminus with an amide improved the peptide's stability in biological media by conferring stability to proteolysis and conferred dipeptidyl carboxypeptidase resistance to the peptide. However, some of the resultant analogs lost a significant amount of their affinity with MHC molecules, as well as immunogenicity and antigenicity. Interestingly, although the three analogs (Table 7) disclosed in this application lost their binding capability with MHC molecule, SSX-2_{41-49} –NH2 (A42V) retained its reactivity with wild-type peptide specific CTLs as indicated by its capability of inducing the secretion of IFN-γ at a similar concentration to that of the wild-type peptide. SSX-2_{41-49} –NH2 (A42L) was, however, able to stimulate the IFN-γ production at a lower concentration.

EXAMPLE 20

DECAMERS

[0234] The length of typical MHC binding peptides varies from 8-mer to 11-mer, and most HLA-A*0201 binding peptides are 9-mers or 10-mers. In previous observations, a 9-mer and 10-mer from a natural sequence were both found to possess a binding motif for the same MHC, and had the same N-terminus. From the standpoint of proteasomal processing they are distinct epitopes, but were nonetheless antigenically cross-reactive. In the case of the wild-type epitope SSX-2_{41-49}, the epitope is a 9-mer peptide and the 10-mer peptide, SSX-2_{41-50}, lacks the appropriate MHC binding motif and showed no immunological activity. Thus, the wild-type epitope was therefore lengthened to a 10-mer with amino acids that could create the appropriate binding motif. As shown in Table 8, while many 10-mer analogs have a lower binding affinity with the HLA-A*0201 molecule, analogs SSX-2_{41-50} (A42L, Y50L/V/Nle/Nva) showed improved binding affinity with the HLA-A*0201 molecule. Two 10-mer analogs in particular, A42L and Y50 Nle/Nva, were able to induce IFN-γ

production from T cells immunized against the wild-type peptide at lower concentrations than the wild-type peptide.

EXAMPLE 21

USE OF ANALOGS TO OVERCOME TOLERIZATION

[0235] One aspect in which the analogs can represent an improvement over the wild-type epitope is in increased immunogenicity in a human system and tolerance breaking. Differences in the TCR repertoire, whether due to germ line differences or differences in negative selection, have the potential to give anomalous results. To address such issues the analogs are used in an *in vitro* immunization of HLA-$A2^+$ blood to generate CTL. Techniques for *in vitro* immunization, even using naive donors, are known in the field, for example, Stauss et al., *Proc. Natl. Acad. Sci. USA* 89:7871-7875, 1992; Salgaller et al. *Cancer Res.* 55:4972-4979, 1995; Tsai et al., *J. Immunol.* 158:1796-1802, 1997; and Chung et al., *J. Immunother.* 22:279-287, 1999; each of which is hereby incorporated by reference in their entirety.

[0236] Specifically, PBMCs from normal donors were purified by centrifugation in Ficoll-Hypaque from buffy coats. All cultures were carried out using autologous plasma (AP) to avoid exposure to potential xenogeneic pathogens and recognition of FBS peptides. To favor the *in vitro* generation of peptide-specific CTL, autologous dendritic cells (DCs) were employed as APCs. DCs were generated and the CTLs were induced with DCs and peptides from PBMCs as described in Keogh et al., 2001, which is incorporated herein by reference in its entirety. Briefly, monocyte-enriched cell fractions were cultured for 5 days with GM-CSF and IL-4 and were cultured for 2 additional days in culture media with 2 μg/ml CD40 ligand to induce maturation. 2 $x10^6$ CD8+-enriched T lymphocytes/well and 2 $x10^5$ peptide-pulsed DCs/well were co-cultured in 24-well plates in 2 ml RPMI supplemented with 10% AP, 10 ng/ml IL-7 and 20 IU/ml IL-2. Cultures were restimulated on days 7 and 14 with autologous irradiated peptide-pulsed DCs. Immunogenicity was assayed using the *in vitro* cytotoxicity and cytokine production assays described herein.

EXAMPLES 22-30

TESTING OF NY-ESO-$1_{157-165}$ ANALOGS

[0237] The analogs listed in Figure 13 were tested for activity, such as binding and biological effect as follows in Examples 22-30:

EXAMPLE 22

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT A SINGLE POSITION (FIGURES 13 A-C).

[0238] The strategy from above (Example 6) was applied to scan through a library of analogs bearing single substitutions relative to the wild-type NY-ESO-$1_{157-165}$ epitope in its native (or

wild-type) version (Figure 13). Strong inverse correlation was found between the minimal required amount of analog to elicit IFN-gamma production *ex vivo* and the maximal amount of cytokine production at any concentration of analog.

[0239] Substitution of S157 with F or K, resulted in analogs that partially retained MHC binding and cross-reactivity with the T cells specific for the nominal epitope. Substitution of L158 with I improved the immunologic features of the peptide as assessed by this methodology; whereas L158V resulted in partial retention of activity. Modification of C165 with any of the amino acids V, L, A, or I resulted in improved immune properties.

[0240] Peptides that have substitutions in the N-terminal position or elsewhere, and present with retained but not increased activity in this assay relative to the wild-type peptide, can be useful in humans. In addition, they are material for further derivatization to improve on their properties, as described below.

EXAMPLE 23

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT TWO POSITIONS (FIGURE 13A-C).

[0241] The strategy from above (Example 6) was applied to scan through a library of analogs bearing two substitutions relative to the wild-type NY-ESO-$1_{157\text{-}165}$ epitope. Simultaneous semi-conservative modifications at position 2 and 9 were shown to have profound effects on the immune properties of analogs, depending on the precise identity of the analogs. Combining L158I with C165V or C165L further increased their activity relative to the wild-type peptide. Similarly, L158V improved on the activity of the C165V or C165L analogs, further increasing such activity relative to wild-type peptide. L158V partially retained the activity of C165A or C165I analogs, showing an interesting effect of double mutation of primary anchor residues. Similarly, L158I partially retained the activity of the C165A analog.

[0242] Simultaneous modifications at positions 1 and 9 had profound effects on the immune properties of analogs, depending on the precise identity of the analogs. S157Y combined with C165Nva (norvaline) or Nle (norleucine) at position 9 resulted in substantially improved activity over S157Y alone or the wild-type peptide. The C165V mutant also rescued the activity of the S157Y mutant. V-NH2 or L-NH2 at position 9 partially rescued the activity of the S157Y analog – however, A-NH2 failed to do so. Combinations between S157F and V, L, I, and to a lesser extent A at the 9th position retained strong activity of the analog and may be more useful than single mutants at position 9 due to the participation of the first residue in the interaction with TCR. Combinations between S157K and V, L, I and to a lesser extent A at the 9th position, retained strong activity of the analog and may be more useful than single mutants at position 9 due to the participation of the first residue in the interaction with TCR and the overall beneficial effect on the peptide solubility of K at position 1.

EXAMPLE 24

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT MULTIPLE POSITIONS (FIGURE 13A-C).

[0243] The strategy from above (Example 6) was applied to scan through a library of analogs bearing multiple substitutions relative to the wild-type NY-ESO-1 epitope.

[0244] L158Nva or L158Nle considerably improved on the activity of the S157Y C165V mutant. Combinations between V or I at position 158 and V, L, A or I at 165 can partially restore the potency of analogs relative to the wild-type peptide. S157Y L158I C165V displayed increased activity relative to the wild-type peptide and S157V with C165V or C165I; and S157I with C165L or I, retained MHC binding and cross-reactivity with T cells specific for the wild-type peptide.

[0245] Triple substitutions comprising Y and V at positions 157 and 165, respectively, in addition to L or N at 160; A, L, V, or N at 162; or E, D or T at 164, retained the activity of the peptide in this cross-reactivity assay, making these analogs useful compounds for breaking T cell tolerance *in vivo* since positions 160, 162 and 164 participate in the interaction with TCR.

[0246] Triple substitutions comprising 157F and 158V plus V, L, I at the position 165 showed activity in the assay described in Example 2. In addition, triple mutants encompassing S157F and L158I plus V or A at position 165 retained activity. Together, these data underline the complex interactive and non-linear nature of multiple substitutions.

[0247] Finally, triple mutants comprising S157W and to a higher extent S157T together with 158V and 165V, showed retained or increased activity respectively, relative to the wild-type peptide.

EXAMPLE 25

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF DECAMERS ENCOMPASSING THE WILD-TYPE PEPTIDE AND MUTATED AT VARIOUS POSITIONS (FIGURE 13A-C).

[0248] The strategy from above (Example 6) was applied to scan through a library of analogs of a decamer encompassing the nominal NY-ESO-$1_{157-165}$ peptide. While the decamer itself lacked significant *in vitro* activity, various substitutions at this position partially rescued activity, such as L at 166, or L, I, Nle at 166 combined with Y at 157 and V at 165 .

[0249] Peptide analogs with similar or reduced activity *in vitro* (but with retained cross-reactivity) compared to the wild-type peptide are still useful for induction or boost of immune responses due to: i) more limited AICD (antigen-induced cell death); ii) higher *in vivo* activity due to increased stability on class I MHC and/or slightly modified interaction with TCR. Thus, these analogs are useful for breaking tolerance.

EXAMPLE 26

EVALUATION OF IMMUNOLOGIC PROPERTIES OF ANALOGS: PEPTIDE BINDING TO MHC CLASS I MOLECULES (FIGURE 13A-C)

[0250] The affinity of peptide analogs and the wild-type epitope to HLA-A*0201 was evaluated by T2 cell based assay (ref. Regner M, et al., Exp Clin Immunogenet. 1996;13(1):30-5), which is incorporated herein by reference in its entirety. For the binding assay, in brief, T2 cells, that lacked expression of TAP and thus do not assemble stable MHC class I on the cell surface, were pulsed with different concentrations of peptides (controls or analogs) overnight at 37°C, washed extensively, stained with fluorescently tagged antibody recognizing MHC class I (A2 allele) and run through a FacsScan analyzer. Peptides that bind A2 stabilize its presence at the cell surface. The difference between the MFI (mean fluorescence intensity) corresponding to a given concentration of analog and the negative control (a non-MHC binding peptide) is a function of how many stabilized complexes between MHC and peptide are displayed on the surface of T2 cells. Thus, at limiting concentrations of peptide, this is a measurement of K_{on} mostly and at saturation levels of peptide that is a measurement of both K_{on} and K_{off}. In Figure 13 the binding is quantified by two factors that are mathematically related: Half maximal binding (the peptide concentration giving 50% of the signal corresponding to saturation) and relative affinity (1/RA), that is binding normalized to a reference (wild-type peptide) – i.e. the ratio between half maximal binding of control relative to peptide analog. The higher the 1/RA index and the lower the half maximal binding, the higher the K_{on} of the interaction between an analog and the MHC molecules. In Figure 13, there are 39 analogs described with such binding parameters improved relative to the wild-type peptide. Such improved binders carry one, two, three, or more substitutions of standard and/or non-standard amino acids at positions that are known to participate in the interaction with MHC and/or TCR, with an overall effect on MHC binding that is dependent on precise / conjugated modification. Such peptide analogs are useful in therapeutic compositions or as a platform to further derive therapeutic compositions.

EXAMPLE 27

METHOD OF IMMUNIZATION (FIGURE 14)

[0251] Eight groups of mice (n=4) were immunized with a plasmid expressing the wild-type NY-ESO-$1_{157\text{-}165}$ epitope, by direct inoculation into the inguinal lymph nodes with 25ug in 25ul of PBS into each lymph node at days 0, 3, 14 and 17. This was followed by two peptide boosts (similar amount) at day 28 and 31, using a negative control peptide (HBVc), wild-type peptide or analog as shown in Figure 14.

EXAMPLE 28

USE OF ANALOGS TO TRIGGER ENHANCED IMMUNITY AGAINST WILD-TYPE EPITOPE, ASSESSED *IN VIVO* (FIGURES 15A-C)

[0252] To evaluate the *in vivo* responses obtained against the wild-type epitope, splenocytes were isolated from littermate control HHD mice and incubated with 20μg/mL or 1μg/ml of wild-type peptide for 2 hours. These cells were then stained with $CFSE^{hi}$ and $CFSE^{med}$ fluorescence (4.0μM or 1μM, respectively, for 15 minutes) and intravenously co-injected into immunized mice with an equal number of control splenocytes stained with $CFSE^{lo}$ fluorescence (0.4μM). Eighteen hours later the specific elimination of target cells was measured by removing the spleens and PBMC from challenged animals and measuring CFSE fluorescence by flow cytometry. The relative depletion of the populations corresponding to peptide loaded splenocytes was calculated relative to the control (unloaded) population and expressed as % specific lysis. Figure 15A shows the lack of *in vivo* cytotoxicity in mice receiving the negative control peptide. Figure 15B shows the variable cytotoxicity in mice immunized with plasmid and amplified with wild-type peptide. Figure 15C shows the substantial, constant cytotoxicity in mice immunized with plasmid and amplified with the analog L158Nva C165V.

EXAMPLE 29

COMPARISON OF VARIOUS ANALOGS IN TRIGGERING ENHANCED IMMUNITY AGAINST THE WILD-TYPE EPITOPE, ASSESSED *IN VIVO* (FIGURES 16A-B)

[0253] In the context of the immunization protocol described above (Example 8) and using the methodology described in the Example 9, *in vivo* cytotoxicity against target cells coated with limited (1μM; Fig.16A) or supraoptimal amounts of wild-type peptide (20μM, Fig. 16B) was evaluated subsequent to the entrain and amplify protocol using plasmid and peptide analog respectively for the two stages. Results expressed as average % specific lysis +/- SE showed that the analog L158V C165Nva induced the highest activity and that the analogs L158V C165V, L158V C165Nva and S157K L158V C165V showed an effect in the same range with wild-type peptide or the C165V mutant. Since multiple substitutions may alter the TCR binding site, such analogs can be more useful than the wild-type peptide in breaking tolerance against a self epitope. In addition, the S157K triple mutant can ameliorate the poor solubility of the wild-type peptide or other analogs, with direct practical implications.

EXAMPLE 30

USE OF ANALOGS TO TRIGGER ENHANCED IMMUNITY AGAINST THE WILD-TYPE EPITOPE, ASSESSED *EX VIVO* BY CYTOKINE PRODUCTION (FIGURES 17A-B)

[0254] In the context of the immunization protocol described above (Example 27), and following the challenge described in Example 28, splenocytes were isolated, pooled and stimulated *in*

vitro with 10μM of wild-type peptide NY-ESO-$1_{157-165}$ for 3 and 6 days respectively. Supernatants were harvested and the concentration of IFN-γ measured by ELISA. Analog L158Nva C165V induced T cells that produced large levels of IFN-gamma more rapidly upon *ex vivo* stimulation (Fig. 17A). Other analogs such as S157F L158V C165V, L158V C165Nva, and L158V C165V induced T cells that produced increased amounts of IFN-gamma upon *ex vivo* re-stimulation with wild-type peptide (Fig. 17B). In contrast, C165V failed to induce increased capability of T cells to produce IFN-γ, relative to the wild-type peptide following the protocols described in Examples 27-28.

EXAMPLE 31

CHARACTERIZATION OF BINDING AND STABILITY BY ELISA (ITOPIA TESTING)

[0255] Avidin-coated microtiter plates containing class I monomer loaded with a so-called placeholder peptide were used to evaluate peptide binding, affinity and off-rate. The monomer-coated plates were supplied as part of the iTopia Epitope Discovery System Kit (Beckman Ciulter, Inc., San Diego, CA, USA). Assay buffers, anti-MHC-FITC mAb and beta2-microglobulin and control peptides were also supplied with the kits.

Binding assay:

[0256] Native peptide and analogs were first evaluated for their ability to bind each MHC molecule by binding assay. This assay measures the ability of individual peptides to bind HLA molecules under standardized optimal binding conditions. Monomer-coated plates were first stripped, releasing the placeholder peptide and leaving only the MHC heavy chain bound to the plate. Test peptides were then introduced under optimal folding conditions, along with the anti-MHC-FITC mAb. Plates were incubated for 18hrs at 21 °C. The anti-MHC-FITC mAb binds preferentially to a refolded MHC complex. Therefore, the fluorescence intensity resulted from each peptide was related to the peptide's ability to form complex with MHC molecule. Each peptide's binding was evaluated relative to the positive control peptide provided in the kit, and the results were expressed '% binding'. The analogs identified as 'better-binders' in relative to the native peptide were subsequently analyzed in the affinity and off-rate assays.

Affinity assay:

[0257] For the affinity assay, after the initial stripping of the placeholder peptide, increasing concentrations (range 10^{-4} to 10^{-8} M) of each test peptides for a given allele were added to a series of wells and incubated under the conditions described previously. Plates were read on the fluorometer. Sigmoidal dose response curves were generated using Prism software. The amount of peptide required to achieve 50% of the maximum was recorded as ED_{50} value.

Off-rate assay:

[0258] For the off-rate assay, the plates were washed after 18 hrs incubation at 21 °C to remove the excess amount peptide. The plates were then incubated on the allele-specific monomer plates at 37 C. The plates were measured at multiple time points (0, 0.5, 1, 1.5, 2, 4, 6 and 8 hrs) for relative fluorescence intensity. The time required for 50% of the peptide to dissociate from the MHC monomer is defined as the T1/2 value (hrs).

iScore calculation:

[0259] The iScore is a multi-parameter calculation provided within the iTopia software. Its value was calculated based on the binding, affinity and stability data.

EXAMPLE 32

VALIDATION OF THE ANTIGENICITY OF $PSMA_{288-297}$

[0260] HHD transgenic mice (n=4) were immunized with $PSMA_{288-297}$ peptide (25μg in 25μl of PBS, plus 12.5μg of pI:C to each lymph node) at day 0, 3, 14 and 17. One week after the boost, splenocytes were stimulated *ex vivo* with the native $PSMA_{288-297}$ peptide and tested against ^{51}Cr-labeled human tumor cells ($PSMA^+$ $A2^+$ LnCap cells, or as negative control, LnCap cells coated with MHC class I-blocking antibody) at various E:T ratios. The results expressed as % specific lysis (mean±SEM), showed that PSMA – specific T cells were able to lyse human tumor cells in a fashion dependent on MHC class I availability, confirming display of the PSMA epitope on MHC class I of tumor cells, in a fashion allowing immune mediated attack (Figure 18).

Examples 33-38

TESTING OF $PSMA_{288-297}$ ANALOGS

[0261] The analogs listed in Figures 19 and 20 were tested for various properties such as improved affinity and stability of binding, cross-reactivity with the native epitope, and immunogenicity as follows in Examples 33-38.

EXAMPLE 33

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT SINGLE POSITION

[0262] Using the procedures described in Example 31 the binding characteristics of $PSMA_{288-297}$ and analogs were assessed in comparison to each other (see Figure 19). The positive control for binding was melan-A_{26-35} A27L. Cross reactivity with the native epitope was assessed by using the analog peptides to stimulate IFG-gamma secretion from a T cell lines specific for the native epitope, essentially as described in example 6. The data shown in Figure 19 was generated by stimulating with 10μg/ml of analog (approximately 10μM). This concentration generally resulted in

maximal or near-maximal IFN-gamma production for the analogs and thus was chosen to represent cross-reactivity.

[0263] The observed affinities of the analogs are reported in Figure 19 as ED50s. Met, Ile, Gln, Val, Nva, Nle, and Abu were substituted at the P2 position. These generally resulted in similar affinity. The Nle and Met substitutions also maintained similar stability of binding, measured as half-time of dissociation in hours. The Val, Nva, and Abu analogs elicited a similar level of IFN-gamma production.

[0264] Val, Leu, Nva, and Nle were substituted for the Ile at the PΩ primary anchor position. All four had similar binding affinity. The Val and Nva substitutions improved the stability of binding and increased the amount of IFN-gamma produced, indicating cross-reactivity and that the analogs can have improved immunogenicity.

[0265] The Ser, Sar, and Abu substitutions at P1 maintained similar binding characteristics but had marginally similar cross-reactivity. The Ala, Leu, Ser, and Thr substitutions at the PΩ-1 position also maintained similar binding characteristics. Finally the Trp substitution at P3 exhibited affinity and stability of binding that were both increased about twofold and IFN-gamma production that was within twofold of the native peptide, all generally similar values.

EXAMPLE 34

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT TWO POSITIONS

[0266] The pattern seen above, that substitutions in this epitope did not greatly impair binding affinity, continued with the double substitutions examined (Figure 20) which uniformly displayed similar or improved binding affinity compared to the native peptide. Among the analogs with substitutions at both primary anchor positions those with Nva of Nle at P2 and Val at PΩ, and Val at P2 and Nva at PΩ displayed improved binding stability and the former two increased IFN-gamma production (data not available for the 3rd analog). The Val and Nva substitutions at PΩ were also paired with Ala and Abu substitutions at P1. These analogs all had robust binding stability and IFN-gamma production that was improved compared to the single PΩ substitutions, thus further improving the P1 substitutions. The PΩ Nva substitution was also able to restore similar cross-reactivity to the P3 Trp substitution.

EXAMPLE 35

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT THREE POSITIONS

[0267] Triple substitutions as P1, P2, and P3; P1, P2, and PΩ; P2, P3, and PΩ; and P1, P3, and PΩ were made (Figure 21). In all cases the P1 substitution was Ala, the P3 substitution was Trp, and the PΩ substitution Val or Nva. As above affinity at least similar to the native peptide was

maintained. For the P1, P2, P3 class Nva and Nle at P2 improved the stability of binding. This P2 Nva analog elicited a similar amount of IFN-gamma while the Nle analog showed a substantial increase.

[0268] For the P1, P2, PΩ class, Nva and Val at P2 and PΩ in either combination improved binding stability. This P2 Nva PΩ Val analog also showed a substantial increase in IFN-gamma production. (No data on the other). Val at both P2 and PΩ in this triple substitution showed binding stability and IFN-gamma production that was nearly halved from that of the native peptide.

[0269] For the P2, P3, PΩ group only the Nva/W/V analog showed improved binding or IFN-gamma production. For the two P1, P3, PΩ analogs examined PΩ of Val or Nva improved binding stability but poor cross-reactivity.

EXAMPLE 36

CROSS-REACTIVE IMMUNOGENICITY OF VARIOUS ANALOGS

[0270] Groups of HHD transgenic mice (n=8) were immunized with peptide (natural epitope $PSMA_{288-297}$, or analogs bearing substitutions at primary or secondary anchor residues), by direct inoculation into the inguinal lymph nodes, with 25μg in 25μl of PBS + 12.5μg of pI:C to each lymph node at day 0, 3, 14 and 17.

[0271] Mice were sacrificed at 10 days after the last boost, splenocytes prepared and assessed for IFN-γ production by ELISPOT analysis. Various numbers of splenocytes / well were stimulated with 10μg/ml of native peptide in ELISPOT plates coated with anti-IFN-γ antibody. At 48 hours after incubation, the assay was developed and the frequency of cytokine-producing T cells that recognized native $PSMA_{288-297}$ peptide was automatically counted. The data were represented in Figure 22 as the number of spot forming colonies / well (mean of triplicates ± SD). The data show increased priming of immune responses against the native epitope achieved by the I297V and P290W analogs, with the other analogs showing slightly higher (but significant) activity than the native peptide (I297Nva or G288Abu or L289Nle I297Nva). To the extent that the poor immunogenicity of the native epitope reflects tolerance, the improved activity of these analogs represents tolerance breaking.

EXAMPLE 37

AMPLIFICATION BY THE I297V ANALOG OF THE RESPONSE TO $PSMA_{288-297}$ INDUCED BY PLASMID

[0272] Two groups of HHD transgenic mice (n=8) were immunized with plasmid expressing $PSMA_{288-297}$, by direct inoculation into the inguinal lymph nodes with 25μg in 25μl of PBS to each lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (25μg) at day 28 and 31 with either the natural peptide or the I297V analog.

[0273] Mice were sacrificed at 10 days after the last boost, splenocytes prepared and assessed for IFN-γ production by ELISPOT analysis. Various numbers of splenocytes / well were stimulated with 10ug/ml of native peptide in ELISPOT plates coated with anti-IFN-γ antibody. At 48 hours after incubation, the assay was developed and the frequency of cytokine-producing T cells that recognized the $PSMA_{288-297}$ peptide was automatically counted. The data were represented in Figure 23 as frequency of specific T cells normalized to 0.5 million responder cells (mean of triplicates + SD). The data show that irrespective of the number of splenocytes / well, the frequency of native epitope – specific T cells was considerably higher in the mouse group immunized with the I297V analog.

EXAMPLE 38

EX VIVO CYTOTOXICITY AGAINST HUMAN TUMOR CELLS

[0274] HHD transgenic mice (n=4) were immunized with plasmid expressing the $PSMA_{288-297}$ epitope, by direct inoculation into the inguinal lymph nodes with 25μg in 25μl of PBS to each lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (same amount) at day 28 and 31, with the analog I297V. One week after the boost, splenocytes were stimulated *ex vivo* with the native $PSMA_{288-297}$ peptide and tested overnight against ^{51}Cr-labeled human tumor cells (Lncap, $A2^+$ $PSMA^+$; or 624.38 $A2^+$ $PSMA^-$ or control 624.28 cells $A2^-PSMA^-$) at various E:T ratios. The resulting immunity was effective in mediating cytotoxicity against Lncap (Figure 24).

EXAMPLE 39

VALIDATION OF THE ANTIGENICITY OF $PRAME_{425-433}$

[0275] HHD transgenic mice (n=4) were immunized with $PRAME_{425-433}$ peptide (25μg in 25μl of PBS, plus 12.5μg of pI:C to each lymph node) at day 0, 3, 14 and 17. One week after the boost, splenocytes were stimulated *ex vivo* with the native $PRAME_{425-433}$ peptide and tested against ^{51}Cr-labeled human tumor cells ($PRAME^+$ $A2^+$ 624.38 melanoma cells; or negative control 624.38 cells, deficient in A2 expression) at various E:T ratios. The results expressed as % specific lysis (mean±SEM), showed that PRAME-specific T cells were able to lyse human tumor cells, confirming display of the $PRAME_{425-433}$ epitope on MHC class I of tumor cells, in a fashion allowing immune mediated attack (Figure 25).

Examples 40-48

TESTING OF $PRAME_{425-433}$ ANALOGS

[0276] The analogs listed in Figures 26-28 were tested for various properties such as improved affinity and stability of binding, cross-reactivity with the native epitope, and immunogenicity as follows in Examples 40-48. Using the procedures described in Example 31 the

HLA-A*0201 binding characteristics of $PRAME_{425-433}$ and 69 analogs were assessed in comparison to each other. The positive control for binding was melan-A_{26-35} A27L. The observed affinities of the analogs are reported as % binding (compared to the positive control) and ED50s. Stability of binding as half time of dissociation. Cross reactivity with the native epitope was assessed by using the analog peptides to stimulate IFG-gamma secretion from a T cell lines specific for the native epitope, essentially as described in Example 6. The data shown in Figures 26-28 were generated by stimulating with analog peptide at approximately 0.3μM. The results were collected from three separate experiments and were normalized to the amount of IFN-γ elicited by the native peptide in each. In some cases the reported values are the average of two determinations. An asterisk "*" indicates that IFN-γ production was not distinguishable from background.

EXAMPLE 40

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT A SINGLE POSITION (FIGURE 26)

[0277] Single substitutions of Val, Met, Ile, Nle, Nva, and Abu were made for the Leu at the P2 primary anchor position. All of these analogs exhibited % binding within 20% of the native peptide. The ED50 was determined for the Met and Nva analogs. The former had an affinity somewhat improved but comparable to the native peptide while the latter's affinity was reduced about 3-fold, but was still comparable to the $PSMA_{288-287}$ epitope. All of the P2 substitutions maintained binding stability at least similar to the native peptide. The Met, Nle, and Nva analogs elicited IFN-γ production within twofold of the native peptide and the Val analog somewhat less.

[0278] Single substitution of Lys, Phe, Tyr, Thr, Orn (ornithine), and Hse (homoserine) were made for the Ser at the P1 position. All of these analogs exhibited % binding within 20% of the native peptide except for the Phe analog which exceeded that range on the high side. The ED50 for the Lys analog has not been determined, but the other five analogs had affinities within threefold of the native peptide. Stability of binding was generally similar to the native peptide with the Phe P1 analog showing greatest binding stability in this group with a half time of dissociation of 17.7 hours compared to 12.2 hours for the native peptide. With the exception of the Lys P1 analog, which elicited 40% of the IFN-γ of the native peptide, all of these analogs were considered cross-reactive as they elicited IFN-γ production within twofold of the native peptide.

[0279] Single substitutions of Val, Ile, Ala, Nle, Nva, Abu, were made to the PΩ anchor position, as well as modifying the carboxy-terminus by the addition of an amide group. Measured binding affinities were at least similar to native peptide. Stability of binding was also generally similar with outliers of the Nva analog at the high end, t1/2 of 17.2 hours, and the C-terminal amide at the low end with a significantly reduced t1/2 of only 3 hours. The Val, Ile, Ala, and Abu PΩ analogs exhibit less preferred cross-reactivity, but the others elicited IFN-γ production within twofold of the native peptide.

[0280] Single substitutions at positions primarily affecting TCR interactions were also made: Nle, Nva, and Abu at P3 and P6, and Ala, Ser, and Sar at P8. The P6 Nva analog produced IFN-γ within twofold of that of the native peptide, though the P6 Abu analog was close at 44%.

EXAMPLE 41

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT TWO POSITIONS

[0281] Double substitution analogs were created at P1 and P2, P2 and PΩ, and P1 and PΩ using various combinations of the single substitutions above (Figures 27A and 27B). None of the P1-P2 double substitutions examined had radical changes to binding affinity or stability, but none of the exhibited significant cross-reactivity in the IFN-γ assay. A similar pattern is seen with the P2-PΩ double substitution analogs, however, the L426Nva L433Nle analog shows a significant level of cross-recativity with the native peptide in the IFN-γ assay along with its similar, somewhat improved binding characteristics. Finally for the P1-PΩ double substitutions, the examined analogs also conformed to the general pattern of having at least similar binding characteristics, but eliciting negligible IFN-γ in the cross-reactivity assay. The exception in this grouping were the S425T L433Nle analog, which exhibited somewhat improved binding stability and significant cross-reactivity, and the S425F L433Nle analog, which had a more that fourfold reduced ED_{50}, a nearly doubled halftime of dissociation, and elicited more IFN-γ than the native peptide.

EXAMPLE 42

CROSS-REACTIVITY AND FUNCTIONAL AVIDITY OF ANALOGS SUBSTITUTED AT THREE POSITIONS

[0282] Four triple substitution analogs were investigated, having Phe or Thr at P1, Nva or Met at P2, and Nle at PΩ. The S425T L426M L433Nle analog had similar affinity whereas the affinity was improved for the other three analogs. Both analogs with P2 Nva substitutions displayed increased stability of binding and significant levels of cross-reactivity. See Figure 28.

EXAMPLE 43

CROSS-REACTIVE IMMUNOGENICITY OF THE L426NVA L433NLE ANALOG

[0283] Two groups of HHD transgenic mice (n=8) were immunized with a plasmid, pCTLR2 described in example 49 below, expressing $PRAME_{425-433}$ by direct inoculation into the inguinal lymph nodes of 25μg in 25μl of PBS to each lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (2.5μg) at day 28 and 31, of native peptide or the PRAME epitope analog L426Nva L433Nle.

[0284] Mice were sacrificed at 10 days after the last boost, splenocytes prepared and assessed for IFN-γ production after *in vitro* stimulation at $0.5x10^6$ cells / well, with 10ug/ml of native peptide. At 48 hours after incubation, the supernatant was harvested and the concentration of IFN-γ produced in response to the $PRAME_{425-433}$ peptide was measured by ELISA. The data were represented in Figure 29 and show a significant enhancement of IFN-γ production in mice boosted with the $PRAME_{425-433}$ L426Nva L433Nle analog.

EXAMPLE 44

IN VIVO CYTOTOXICITY INDUCED BY THE L426NVA L433NLE ANALOG

[0285] Two groups of HHD transgenic mice (n=8) were immunized as described in Example 43 above.

[0286] To evaluate the *in vivo* responses obtained against the native epitope, splenocytes were isolated from littermate control HHD mice and incubated with 20μg/mL or 1μg/ml of native peptide for 2 hours. These cells were then stained with $CFSE^{hi}$ and $CFSE^{med}$ fluorescence (4.0μM or 1μM, respectively, for 15 minutes) and intravenously co-injected into immunized mice with an equal number of control splenocytes stained with $CFSE^{lo}$ fluorescence (0.4μM). Eighteen hours later the specific elimination of target cells was measured by removing the spleens and PBMC from challenged animals and measuring CFSE fluorescence by flow cytometry. The relative depletion of the populations corresponding to peptide loaded splenocytes was calculated relative to the control (unloaded) population and expressed as % specific lysis. The results in Figure 30 showed preserved induction of cytotoxicity when the analog replaced the natural peptide as a booster agent. The trend indicates that the analog can improve on induction of cytotoxic immunity.

EXAMPLE 45

IN VIVO CYTOTOXICITY AND TETRAMER STAINING

[0287] Seven groups of HHD transgenic mice (n=4) were immunized with a plasmid, pCTLR2, expressing $PRAME_{425-433}$ by direct inoculation into the inguinal lymph nodes of 25μg in 25μl of PBS to each lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (2.5μg) at day 28 and 31, of native peptide, negative control (EAAGIGILTV peptide), or $PRAME_{425-433}$ epitope analogs bearing mutations at the primary and/or secondary anchor residues – S425F, L426Nva L433Nle, S425T L433Nle, and S425T L426Nva L433Nle.

[0288] To evaluate the *in vivo* response against native peptide, splenocytes were isolated from littermate control HHD mice and incubated with 0.2ug/ml or 20ug/ml of native peptide for 2 hours. These cells were then stained with CFSE fluorescence (1 and 2.5μM respectively, for 15 minutes) and intravenously co-injected into immunized mice with an equal number of control splenocytes stained with $CFSE^{lo}$ fluorescence (0.4μM). Eighteen hours later the specific elimination

of target cells was measured by removing the spleen from challenged animals and measuring CFSE fluorescence in the resulting cell suspensions, by flow cytometry. The relative depletion of the populations corresponding to peptide-loaded splenocytes was calculated relative to the control (unloaded) population and expressed as % specific lysis. In addition, the frequency of $PRAME_{425-433}$-specific T cells, was evaluated by tetramer / CD8 co-staining. The boost with analogs encompassing mutations at primary or secondary anchor residues showed comparable immune activity as compared to the native peptide, based on *in vivo* cytotoxicity and tetramer staining. The analogs were capable of amplifying the immune response as shown by comparison with the "EAA" group, boosted with an irrelevant peptide. In that regard, analogs comprising S425F, L33Nle, L426Nva L433Nle, S425T L433Nle, or S425T L426Nva L433Nle were all capable of expanding the immunity against the native epitope, as assessed by in vivo cytotoxicity. However, only the L433Nle, L426Nva L433Nle, and S425T L426Nva L433Nle analogs expanded the subset of T cells specific against the native epitope to a level significantly higher that in mice primed with plasmid and boosted with the negative control peptide (Figure 31).

EXAMPLE 46

EX VIVO CYTOKINE PRODUCTION

[0289] Three groups of HHD transgenic mice (n=4) were immunized with a plasmid, pCTLR2, expressing $PRAME_{425-433}$ by direct inoculation into the inguinal lymph nodes of 25μg in 25μl of PBS to each lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (2.5μg) at day 28 and 31, of the PRAME epitope analogs L426Nva L433Nle and S425T L426Nva L433Nle or the negative control peptide Melan A (EAAGIGILTV).

[0290] Mice were sacrificed at 10 days after the last boost, splenocytes prepared and assessed for IFN-γ production by ELISA at 48 hours after incubation with 10μg/ml of native peptide. The data were represented in Figure 32 as cytokine concentration in pg/ml (mean of triplicates ± SD). The data showed *ex vivo* cytokine production by splenocytes from mice boosted with both analogs, and greater response to L426Nva L433Nle than to S425T L426Nva L433Nle.

EXAMPLE 47

EX VIVO CYTOTOXICITY AGAINST A HUMAN TUMOR CELL LINE AFTER PEPTIDE BOOST WITH ANALOG

[0291] HHD transgenic mice (n=4) were immunized with a plasmid, pCTLR2, expressing PRAME425-433 by direct inoculation into the inguinal lymph nodes of 25μg in 25μl of PBS to each lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (2.5μg) at day 28 and 31, with the analog L426Nva L433Nle. One week after the boost, splenocytes were stimulated *ex vivo* with the native peptide and tested against ^{51}Cr-labeled human tumor cells

(PRAME$^+$ 624.38 melanoma cells pretreated or not with IFN-γ; or negative control 624.38 cells, deficient in HLA-A2 expression) at various E:T ratios. The analog L426Nva L433Nle elicited immune responses that mediated significant cytotoxicity against human tumor cells expressing A2 (624.38), slightly elevated upon their pre-treatment with IFNγ. In contrast, no significant activity was measured against A2- 624.28 control cells. See Figure 33.

EXAMPLE 48

IN VITRO IMMUNIZATION TO PRAME425-433

[0292] *In vitro* immunization was carried out according to the general scheme presented in Figure 34. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (HLA-A*0201$^+$) by Ficoll-separation. Fresh PBMCs (2.5 x 10^6), together with 5 ng/ml $PRAME_{425-433}$ or peptide analog were plated in T-cell culture medium. Subsequently 20 IU/ml of interleukin 2 was added to each well after 72 and 96 hours and addition peptide (5 ng/ml) was added at day 7. Cultures were maintained for an additional 10 days before effector cells were harvested and used in tetramer staining. IVS PBMCs were labeled with $PRAME_{425-433}$ tetramer and analyzed on the FACSCalibur (BD, San Jose, CA). Quadrants were set based on negative controls, stained with irrelevant HBV tetramer and SSX2 tetramer, and a minimum of 10,000 gated events were captured. Tetramer-positive cells are expressed as a percentage of the lymphocyte population. $PRAME_{425-433}$–specific tetramers was significantly enhanced following IVS with peptide analog as compared with native peptide. See Figure 35. This demonstrates that the analog can be a preferable immunogen.

EXAMPLE 49

PCTLR2, A PLASMID EXPRESSING THE $PRAME_{425-433}$ EPITOPE

[0293] pCTLR2 is a recombinant DNA plasmid vaccine which encodes one polypeptide with an HLA A2-specific CTL epitope, SLLQHLIGL, from PRAME amino acid residues 425-433, and an epitope cluster region of PRAME, amino acids 422-509. The cDNA sequence for the polypeptide in the plasmid is under the control of promoter/enhancer sequence from cytomegalovirus (CMVp) which allows efficient transcription of messenger for the polypeptide upon uptake by antigen presenting cells. The bovine growth hormone polyadenylation signal (BGH polyA) at the 3' end of the encoding sequence provides signal for polyadenylation of the messenger to increase its stability as well as translocation out of nucleus into the cytoplasm. To facilitate plasmid transport into the nucleus, a nuclear import sequence (NIS) from Simian virus 40 has been inserted in the plasmid backbone. One copy of CpG immunostimulatory motif is engineered into the plasmid to further boost immune responses. Lastly, two prokaryotic genetic elements in the plasmid are responsible for amplification in E.coli, the kanamycin resistance gene (Kan R) and the pMB bacterial origin of replication. (See Figure 36).

Immunogen Translation Product Sequence

[0294] The amino acid sequence of the encoded polypeptide (150 amino acid residues in length) is given below.

[0295]

malqsllqhliglsnlthvlypvplesyedihgtlhlerlaylharlrellcelgrpsmvwlsanpcphcgdrtfyd pepilcpcfmpnkr**sllqhligl**gdaay**sllqhlig***lispekeeqyia***sllqhligl**krpsikr**sllqhligl** (SEQ ID NO:100).

[0296] The first 89 amino acid residues are an epitope cluster region representing PRAME 422-509. Within this epitope cluster region, a number of potential HLA A2-specific CTL epitopes have been found using a variety of epitope prediction algorithms. Amino acid residues 90-150 are an epitope liberation (synchrotope™) sequence with four copies of PRAME 425-433 CTL epitope (**boldface**) embedded. Flanking the defined PRAME CTL epitope are short amino acid sequences that have been shown to play an important role in the processing of the PRAME CTL epitope. In addition, the amino acid sequence ispekeeqyia (corresponding to PRAME amino acid 276-286, in *italics*) is engineered into the sting-of-beads region to facilitate the detection of expression of encoded polypeptide.

[0297] Using a variety of immunological assays including tetramer, ELISPOT, ELISA, and cytotoxicity, strong CTL responses specific for epitope $PRAME_{425-433}$ have been detected from HLA-A2 transgenic mice immunized with the pCTLR2 plasmid, suggesting immunogenic potency for pCTLR2. These data indicated that the plasmid has been taken up by antigen presenting cells, the encoded polypeptide has been synthesized and proteolytically processed to produce the nonamer epitope peptide, and become HLA-A2 bound for presentation.

Plasmid construction

[0298] Stepwise ligation of sets of long complementary oligonucleotides resulted in generation of cDNA encoding amino acid residues in the "String-of-Beads" epitope liberation sequence (amino acids 90-150). These cDNA bore appropriate cohesive ends for restriction enzymes that can be used for further ligation with cDNA encoding the PRAME epitope cluster region (amino acid 1-89), which were amplified by performing PCR on cDNA encoding PRAME as template. The entire insert was then ligated into vector backbone between *Afl* II and *EcoR* I sites. The entire coding sequence was verified by DNA sequencing.

EXAMPLE 50

GENERATION OF ANTIGEN SPECIFIC T CELL RESPONSES

[0299] H-2 class I-negative, HLA-A2.1-transgenic HHD mice were housed under pathogen-free conditions and used for evaluation of the immunogenicity of HLA-A2.1-restricted human tumor-associated cytotoxic T lymphocyte (CTL) epitopes. Female mice 8-12 weeks of age were used for intralymphatic immunization and for isolation of splenocytes for *in vivo* cytotoxicity studies. The mice were immunized via bilateral inguinal lymph node injection. Mice were anesthetized by inhalation of isofluorane and surgeries were conducted under aseptic conditions. Following preparation for surgery, an incision 0.5 cm in length was made in the inguinal fold and the inguinal lymph node was exposed. A maximum volume of 25 µl (25µg) of plasmid DNA vaccine or peptide was injected directly into the lymph node using a 0.5 mL insulin syringe. The wound was closed with sterile 6-0 nylon skin sutures.

EXAMPLE 51

EX VIVO CYTOTOXICITY AGAINST HUMAN TUMOR CELLS

[0300] HHD transgenic mice (n=4/group) were immunized with the plasmid pSEM (described more fully in U.S. Patent Application No. 10/292,413 (Pub. No.20030228634 A1)) incorporated by reference above in its entirety) expressing melan-$A_{26\text{-}35}$ A27L epitope analog, by direct inoculation into the inguinal lymph nodes with 25ug in 25ul of PBS/each lymph node at day 0, 3, 14 and 17. This was followed by two additional peptide boosts (same amount) at day 28 and 31, with the analogs A27L, A27Nva, or A27L V35Nva. One week after the boost, splenocytes were stimulated *ex vivo* with the native melan-$A_{26\text{-}35}$ peptide and tested against ^{51}Cr-labeled human tumor cells (624.38 cells) at various E:T ratios. The resulting immunity after boosting with the A27L or A27Nva analogs was comprable and more effective than the native peptide EAAGIGILTV (Figure 37). Since the priming plasmid expresses the A27L analog the experiment had a potential bias in favor that peptide, so that the substantial cytotoxicity obtained with the A27Nva analog may be an underestimate of it potency if priming made use of that same sequence.

EXAMPLE 52

TETRAMER ANALYSIS

[0301] Enumeration of CD8+ antigen-specific T cells requires cognate recognition of the T cell receptor (TCR) by a Class I MHC / peptide complex. This can be done using Class I MHC tetramers which are composed of a complex of four HLA MHC Class I molecules each bound to the specific peptide and conjugated with a fluorescent protein. Thus tetramer assays allow quantitation of the total T cell population specific for a given peptide complexed in a particular MHC molecule. Furthermore, since binding does not depend on functional pathways, this population includes all specific CD8+ T cells regardless of functional status. The CTL response in immunized animals was

measured by co-staining mononuclear cells isolated from peripheral blood after density centrifugation (Lympholyte Mammal, Cedarlane Labs) with HLA-A*0201 MART1 (ELAGIGILTV)-PE MHC tetramer (Beckman Coulter, T01008) or a $Tyrosinase_{369-377}$ (YMDGTMSQV) specific tetramer reagent (HLA-A*0201 Tyrosinase-PE, Beckman Coulter) and FITC conjugated rat anti-mouse CD8a (Ly-2) monoclonal antibody (BD Biosciences). Data was collected using a BD FACS Calibur flow cytometer and analysed using cellquest software by gating on the lymphocyte population and calculating the percent of $tetramer^+$ cells within the $CD8^+$ CTL population.

EXAMPLE 53

TETRAMER STAINING (PLASMID PRIMING,
PEPTIDE BOOST – NATIVE VERSUS ANALOG)

[0302] Two groups of HHD transgenic mice (n=8) were immunized with plasmid expressing Tyrosinase 369-377, by direct inoculation into the inguinal lymph nodes with 25ug in 25ul of PBS/each lymph node at day 0, 3, 14 and 17. This was followed by two additional peptide boosts (similar amount) at day 28 and 31, of natural peptide or the 377Nva analog. Ten days later, the immune response was monitored using a Tyrosinase 369-377 specific tetramer reagent (HLA-A*0201 Tyrosinase-PE, Beckman Coulter). Individual mice were bled via the retro-orbital sinus vein and PBMC were isolated using density centrifugation (Lympholyte Mammal, Cedarlane Labs) at 2000rpm for 25 minutes. PBMC were co-stained with a mouse specific antibody to CD8 (BD Biosciences) and the Tyrosinase tetramer reagent and specific percentages were determined by flow cytometery using a FACS caliber flow cytometer (BD). The percentages of Tyrosinase specific $CD8^+$ cells, show that replacement of the native peptide with the analog, preserved the expansion of Tyrosinase-specific subset. The trend indicates that the analog can improve on the expansion of Tyrosinase specific T cells (Figure 38).

EXAMPLE 54

IN VIVO CYTOTOXICITY AND TETRAMER STAINING
(HEAD TO HEAD COMPARISON BETWEEN NATIVE PEPTIDE
AND A PANEL OF ANALOG CANDIDATES)

[0303] Four groups of HHD transgenic mice (n=6) were immunized with plasmid (pSEM) expressing $Tyrosinase_{369-377}$ and Melan-A_{26-35} A27L epitopes, by direct inoculation into the inguinal lymph nodes of 25ug of plasmid in 25ul of PBS per lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (similar amount) at days 28 and 31, of Melan-A_{26-35} A27L into the left inguinal lymph node and $Tyrosinase_{369-377}$ analogs, bearing substitutions at the primary and/or secondary anchor residues, into the right lymph node. As controls, mice immunized with plasmid only or naïve mice were used.

[0304] To evaluate the *in vivo* response against natural Tyrosinase and Melan A epitopes, splenocytes were isolated from littermate control HHD mice and incubated separately, with 20ug/ml of natural peptide (Melan-A_{26-35} or $Tyrosinase_{369-377}$) for 2 hours in HL-1 serum free medium (Cambrex) at a concentration of 20 x 10^6 cells/mL. These cells were then stained with CFSE (Vybrant CFDA SE cell tracer kit, Molecular Probes) (1 and 2.5μM respectively, for 15 minutes) and intravenously co-injected into immunized or naïve control HHD mice with an equal number of control non-peptide coated splenocytes stained with $CFSE^{lo}$ fluorescence (0.4μM). Eighteen hours later the specific elimination of target cells was measured by removing the spleen from challenged animals and measuring CFSE fluorescence in the resulting cell suspensions, by flow cytometry. The relative depletion of the populations corresponding to peptide-loaded splenocytes was calculated relative to the control (unloaded) population and expressed as % specific lysis. In addition, the frequency of $Tyrosinase_{369-377}$- and Melan-A_{26-35}-specific T cells, was evaluated by tetramer / CD8 co-staining (HLA-A*0201-tetramers, Beckman Coulter).

[0305] The tyrosinase analog V377Nva was capable of expanding the population of tyrosinase-specific T cells and amplifying cytotoxic immunity, similarly to the native peptide and greater than the Tyrosinase analog M370V V377Nva (Figure 39).

EXAMPLE 55

EX VIVO CYTOTOXICITY AGAINST HUMAN TUMOR CELLS

[0306] HHD transgenic mice (n=4/group) were immunized (according to the general protocol in Figure 40) with plasmid (pSEM) expressing the $Tyrosinase_{369-377}$ epitope, by direct inoculation into the inguinal lymph nodes of 25ug of plasmid in 25ul of PBS per lymph node at day 0, 3, 14 and 17. This was followed by two peptide boosts (same amount) at day 28 and 31, with the native peptide or analogs bearing substitutions at primary anchor residues P2 and PΩ (370 and 377). One week after the boost, splenocytes were stimulated *ex vivo* with the native $Tyrosinase_{369-377}$ peptide and assayed against ^{51}Cr-labeled human tumor cells (624.38 cells) at various E:T ratios. Both the native peptide and the M370V V377Nva analog generated robust cytotoxicity against 624.38 cells (Figure 41). Whereas there was some dilution of cytolytic activity with the native peptide there was none with the analog reinforcing the indication of greater immunogenicity gained from the tetramer results in Example 52. Together with the preceding example, this observation illustrates the usefulness of complementing more stringent assays (in vivo cytotoxicity and tetramer staining) with more sensitive assays (ex vivo cytotoxicity after in vitro stimulation), to outline potentially useful analogs.

[0307] The various methods and techniques described above provide a number of ways to carry out the invention. Of course, it is to be understood that not necessarily all objectives or advantages described may be achieved in accordance with any particular embodiment described

herein. Thus, for example, those skilled in the art will recognize that the methods may be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as may be taught or suggested herein.

[0308] Furthermore, the skilled artisan will recognize the interchangeability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step, can be mixed and matched by one of ordinary skill in this art to perform methods in accordance with principles described herein. Among the various elements, features, and steps some will be specifically included and others specifically excluded in diverse embodiments.

[0309] Although the invention has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the invention extends beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and obvious modifications and equivalents thereof.

WHAT IS CLAIMED IS:

1. An isolated peptide consisting essentially of a sequence in which:

P0 is X, XX, or XXX, wherein X specifies any amino acid or no amino acid; and

P1 is K, F, Y, W, Phg, Phe(4-F), Phe(4-NO_2), MeTyr, β-(3-benzothienyl)-Ala, or D-Lys; and

P2 is A, L, V, I, M, D-Ala, Nal-2, Abu, Aib, Nle, or Nva; and

P3 is S; and

P4 is E, Q, Nle, or Nva; and

P5 is K: and

P6 is I, L, V, Nle, or Nva; and

P7 is F; and

P8 is Y, F, Phe(4-F); and

PΩ at P9 is V, I, A, Nva, MeVal, Abu, or V-NH_2, or P9 is V, and PΩ at P10 is I, L, V, Nle or Nva; and

PΩ+1 is X, XX, or XXX, wherein X specifies any amino acid or no amino acid; and

wherein the sequence is not KASEKIFYV;

2. An isolated peptide of claim 1 consisting essentially of the sequence:

K{L, V, M, I, D-Ala, D-Val, Nal-2, Aib, Abu, Nle, or Nva}SEKIFYV; or

{F, Phg, Y, Phe(4-F), Phe(4-NO_2), O-methyl-Tyr, or β-(3-benzothienyl-Ala}ASEKIFYV; or

{Y, F, or W}{V, M, or I}SEKIFYV; or

{F or W}LSEKIFYV; or

K{A, V, or L}SEKIFYI; or

K{L or V}SEKIFYV-NH_2; or

FVSEKIFY{I, A, Nva, Abu, or MeVal}; or

FVS{Q, Nle, Nva}KIFYV; or

FVSEK{L, V, Nle, or Nva}FYV; or

FVSEKIF{F, Phe(4-F)}V; or

KASEKIFYV{I, L,};or

KVSEKIFYV {I, L, V, or Nle}; or

KLSEKIFYV {L, V, Nle, or Nva}.

3. An isolated peptide of claim 2 consisting essentially of the sequence:

K{L, V, M, Abu, Nle, or Nva} SEKIFYV; or

{F or Phg}A SEKIFYV; or

YVSEKIFYV; or

F{L, V, or I}SEKIFYV; or

W{L or I}SEKIFYV; or

K{V or L}SEKIFYI.; or

FVSEKIFY{I or Nva}.

4. The isolated peptide of claim 3 consisting essentially of the sequence:

K{V or L}SEKIFYV; or

{F or Y}ASEKIFYV; or

FVSEKIFYI; or

KVSEKIFYV.

5. The isolated peptide of Claim 3, consisting essentially of the sequence KVSEKIFYV.

6. The isolated peptide of claim 1 wherein the peptide has affinity for a class I MHC peptide binding cleft.

7. The isolated peptide of claim 6 wherein the class I MHC is HLA-A2.

8. A class I MHC/peptide complex wherein the peptide has the sequence of the peptide of claim 1.

9. The class I MHC/peptide complex of claim 8 that is cross-reactive with a TCR that recognizes a class I MHC/SSX-2_{41-49} complex.

10. The class I MHC/peptide complex of claim 9 wherein the class I MHC/ complex is an HLA-A2/SSX-2_{41-49} complex.

11. A polypeptide comprising the peptide sequence of claim 1 in association with a liberation sequence.

12. An immunogenic composition comprising the peptide of claim 1.

13. An immunogenic composition comprising the polypeptide of claim 11.

14. A nucleic acid encoding the polypeptide of claim 11.

15. Nucleic acid means for expressing the peptide of claim 1.

16. An immunogenic composition comprising the nucleic acid of claim 14 or 15.

17. A method of inducing, maintaining, or amplifying a CTL response comprising intranodal administration of the composition of claim 12.

18. A method of entraining a class I MHC-restricted T cell response comprising intranodal administration of the composition of claim 12 plus an immunopotentiating agent.

19. A method of inducing, maintaining, or entraining a CTL response comprising intranodal administration of the composition of claim 16.

20. An isolated peptide comprising 1 to 3 substitutions in the sequence KASEKIFYV having an affinity for a class I MHC binding cleft that is similar to or greater than the affinity of KASEKIFYV for said class I MHC binding cleft.

21. The isolated peptide of claim 20 wherein the halftime of dissociation is similar to or greater than the halftime of dissociation of KASEKIFYV from said class I MHC binding cleft.

22. The isolated peptide of claim 20 that is recognized by T cells with specificity for the peptide KASEKIFYV.

23. An isolated peptide consisting essentially of a sequence in which:

P0 is X, XX or XXX, wherein X specifies any amino acid or no amino acid; and

P1 is S, F, K, W or Y; and

P2 is L, I, V, Nle, or Nva; and

P3 is L; and

P4 is M, L, or N; and

P5 is W; and

P6 is I, A, L, V, or N; and

P7 is T; and

P8 is Q, E, D, or T; and

PΩ at P9 is C, V, I, L, A, Nva, Nle, V-NH_2, or L-NH_2; and

PΩ + 1 is X, XX, XXX, wherein X specifies any amino acid or no amino acid; and

wherein the sequence is not SLLMWITQ{C, V, I, L, A}, FVLMWITQA, FILMWITQ{L, I}, YVLMWITL or YLLMWIT{I, L}.

24. The isolated peptide of Claim 23, consisting essentially of the sequence SILMWITQ{C, V, L, A}, YLLMWITQ{Nva, Nle}, F{L, V}LMWITQ{V, L, I}, Y{I, Nva, Nle}LMWITQV, YLLLWITQV, or TVLMWITQV.

25. The isolated peptide of Claim 23, consisting essentially of the sequence {S, F}VLMWITQV, SLMWITQNva, or SNvaLMWITQV.

26. The isolated peptide of Claim 25, consisting essentially of the sequence SNvaLMWITQV.

27. The isolated peptide of Claim 23, wherein the peptide has affinity for a class I MHC peptide binding cleft.

28. The isolated peptide of Claim 27, wherein the MHC is HLA-A2.

29. A class I MHC/peptide complex wherein the peptide has the sequence Claim 23.

30. The class I MHC/peptide complex of claim 29 that is cross-reactive with a TCR that recognizes a class I MHC/NY-ESO-$1_{157\text{-}165}$ complex.

31. The class I MHC/peptide complex of claim 30 wherein the class I MHC/complex is an HLA-A2/NY-ESO-$1_{157\text{-}165}$ complex.

32. A polypeptide comprising the peptide sequence of Claim 23 in association with a liberation sequence.

33. An immunogenic composition comprising the peptide of Claim 23.

34. An immunogenic composition comprising the polypeptide of Claim 32.

35. A nucleic acid encoding the polypeptide of Claim 32.

36. Nucleic acid means for expressing the peptide of claim 23.

37. An immunogenic composition comprising the nucleic acid of Claim 35 or 36.

38. A method of inducing, maintaining, or amplifying a CTL response comprising intranodal administration of the composition of claim 33.

39. A method of inducing, maintaining, or amplifying a CTL response comprising intranodal administration of the composition of claim 37.

40. A method of entraining a class I MHC-restricted T cell response comprising intranodal administration of the composition of Claim 37.

41. A method of entraining a class I MHC-restricted T cell response comprising intranodal administration of the composition of Claim 33 plus an immunopotentiating agent.

42. An isolated decamer peptide having the sequence {S,Y}LLMWITQ{C, V}{L, I, Nle}.

43. An isolated peptide comprising 1 to 3 substitutions in the sequence SLLMWITQC having an affinity for a class I MHC binding cleft that is similar to or greater than the affinity of SLLMWITQC for said class I MHC binding cleft.

44. The isolated peptide of claim 43 wherein the halftime of dissociation is similar to or greater than the halftime of dissociation of SLLMWITQC from said class I MHC binding cleft.

45. The isolated peptide of claim 43 that is recognized by T cells with specificity for the peptide SLLMWITQC .

46. A peptide having an amino acid sequence comprising at least one difference from a sequence of a segment of a target-associated antigen, the segment having known or predicted affinity for the peptide binding cleft of a MHC protein, wherein the at least one difference is a Nle or Nva residue replacing a residue at an MHC-binding motif anchor position in said segment.

47. The peptide of claim 46 wherein the anchor position is a primary anchor position.

48. The peptide of claim 47 wherein said anchor position is P2.

49. The peptide of claim 47 wherein said anchor position is PΩ.

50. The peptide of claim 46 wherein the anchor position is an auxiliary anchor position.

51. The peptide of claim 46 wherein said difference comprises a Nle or Nva residue replacing a hydrophobic residue in said segment.

52. The peptide of claim 46 wherein I, L, or V is a preferred residue in said MHC-binding motif anchor position.

53. The peptide of claim 46 having a length of about 8 to about 14 amino acids.

54. The peptide of claim 53 having a length of 9 to 10 amino acids.

55. The peptide of claim 46 wherein said MHC protein is a human MHC protein.

56. The peptide of claim 46 wherein said MHC protein is a class I MHC protein.

57. The peptide of claim 56 wherein said MHC protein is of a type selected from the group consisting of HLA-A2, A3, A24, A30, A66, A68, A69, B7, B8, B15, B27, B35, B37, B38,

B39, B40, B48, B51, B52, B53, B 60, B61, B62, B63, B67, B70, B71, B75, B77, C4, Cw1, Cw3, Cw4, Cw6, Cw7, and Cw10.

58. The peptide of claim 57 said MHC protein is selected from the group consisting of HLA-A2 and A24.

59. The peptide of claim 46, said MHC having an anchor residue binding pocket, wherein the pocket is homologous to the B- or F-pocket of HLA-A*0201.

60. The peptide of claim 46 having at least one binding characteristic that is substantially the same as, or better than, a corresponding characteristic of said segment for said MHC.

61. The peptide of claim 60, wherein said binding characteristic is elevated compared with that of said segment.

62. The peptide of claim 60 wherein said binding characteristic is affinity.

63. The peptide of claim 60 wherein said binding characteristic is stability of binding.

64. The peptide of claim 46 having an immunogenicity that is substantially the same as, or better than, the immunogenicity of said segment.

65. The peptide of claim 64 wherein said immunogenicity is increased.

66. The peptide of claim 64 wherein said immunogenicity evokes an immune response that is cross-reactive to said segment.

67. The peptide of claim 64 wherein said immunogenicity evokes a CTL response.

68. The peptide of claim 64 wherein said immunogenicity is assessed using an MHC-tetramer assay.

69. The peptide of claim 64 wherein said immunogenicity is assessed using a cytokine assay.

70. The peptide of claim 64 wherein said immunogenicity is assessed using a cytotoxicity assay.

71. The peptide of claim 64 wherein said immunogenicity is assessed by measuring an immune response recognizing the peptide.

72. The peptide of claim 64 wherein said immunogenicity is assessed by measuring an immune response recognizing said segment.

73. The peptide of claim 64 wherein said immunogenicity is assessed using an in vitro immunizations system.

74. The peptide of claim 73 wherein said immunization system comprises human cells.

75. The peptide of claim 64 wherein said immunogenicity is assessed using an in vivo immunization system.

76. The peptide of claim 75 wherein said immunization system comprises a transgenic mouse.

77. The peptide of claim 46 that is immunologically cross-reactive with said segment.

78. The peptide of claim 77 wherein the cross-reactivity is assessed by immunizing with said segment and assaying recognition of the peptide.

79. The peptide of claim 77 wherein the cross-reactivity is assessed by immunizing with the peptide and assaying recognition of said segment.

80. The peptide of claim 46 comprising two differences.

81. The peptide of claim 80 wherein each difference independently comprises a Nle or Nva residue.

82. The peptide of claim 80 wherein one difference does not comprise a Nle or Nva residue.

83. The peptide of claim 46 comprising three or more differences.

84. The peptide of claim 46 wherein the target-associated antigen is a tumor-associated antigen.

85. The peptide of claim 46 wherein the target-associated antigen is a pathogen-associated antigen.

86. An immunogenic composition comprising the peptide of claim 46.

87. A method of immunization comprising administering the composition of claim 67 to a mammal.

88. The method of claim 43 comprising administering directly to the lymphatic system.

89. A method of making a T cell epitope analogue comprising,

providing an amino acid sequence of a segment of a target-associated antigen, the segment having known or predicted affinity for the peptide binding cleft of a MHC protein,

changing at least one amino acid of the sequence corresponding to an anchor position of a MHC binding motif to Nle or Nva, and

synthesizing a peptide comprising the changed sequence.

90. A T cell epitope peptide analogue wherein the analogue differs from a native epitope peptide by replacement of at least one native residue corresponding to an anchor position of a MHC binding motif with a Nle or Nva residue.