(54) Title: IMMUNE RESTRICTED PEPTIDES WITH INCREASED EFFICACY

(57) Abstract: The present invention relates to immune restricted peptides, and especially HLA-A2 restricted peptides. Specifically, the present invention relates to immune restricted peptide according to the general formula: where: 

\[ P_1 \] is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear aliphatic, aromatic or heteroaromatic substitution; 

\[ P_2 \] is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear or branched substitution; 

\[ P_3 \] is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear or branched substitution; 

\[ P_4 \] is a naturally, or non-naturally, occurring amino acid comprising a methylated alpha nitrogen atom; 

\[ P_m \] is a naturally occurring amino acid, \( n \) is an integer of 1 to 9, preferably 1 to 4, more preferably 2 or 3; 

\[ P_{-1} \] is a naturally, or non-naturally, occurring amino acid comprising a fluorescent aromatic substitution; 

\[ P_{c1} \] is a naturally, or non-naturally, occurring amino acid; 

\[ P_{c2} \] is a naturally, or non-naturally, occurring amino acid comprising unsaturated side chains and/or carboxyl isosteres; under the condition that at least one of \( P_1, P_2, P_3, P_n, P_{c1}, P_{c2} \) and \( P_c \) is a non-naturally occurring amino acid.

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— of inventorship (Rule 4.17(iv))
IMMUNE RESTRICTED PEPTIDES WITH INCREASED EFFICACY

Description

The present invention relates to immune restricted peptides, and especially HLA-A2 restricted peptides. Further, the present invention relates to a method for providing the present immune restricted peptides and the use thereof in medicine and especially the use thereof in vaccines, immunosuppressive therapy, adoptive T cell therapy and diagnostics.

Existing and newly emerging diseases that threaten public health demand the development of new technologies combating, or preventing, these diseases. One approach to combat, or prevent, diseases is to use, or direct, the own defence system of a subject, i.e. the immune system, for example by vaccination, immunosuppressive therapy, or adoptive T cell therapy.

A vaccine is a biological preparation that stimulates, activates or improves the response of the immune system towards a particular disease or condition. Vaccines can be prophylactic, for example to prevent or ameliorate the effects of a future infection by a pathogen, or therapeutic, for example vaccines against cancer.

A classical vaccine typically contains an agent that mimics a disease-causing agent such as a microorganism, and is often made from weakened or killed forms of a pathogen.

Besides the above classical vaccines, considerable efforts have been made towards the development of subunit vaccines. Rather than introducing an inactivated or attenuated micro-organism in order to stimulate, activate or improve the immune system, a fragment, such as a peptide, is used.
Peptide vaccines are generally preparations comprising synthetic epitopes in the form of peptides, i.e. short strings of consecutive amino acid forming sequences up to 20, 30, 40 or 50 amino acids, representing one or more minimal immunogenic regions of a protein or antigen.

All nucleated cells present peptides that are derived, or originate, from intracellular proteins on their surface bound to MHC class I, whereas peptides derived, or originate, from extracellular proteins are mainly presented by MHC class II on specialised antigen-presenting cells, APCs, such as dendritic cells and macrophages.

In both cases, the T cell receptor or TCR on the surface of the cytolytic T lymphocyte, CTL, or T_h cell forms a complex with the MHC I/peptide-epitope complex or the MHC II/peptide-epitope complex, respectively; these interactions are aided by the CD8 and CD4 co-receptors, respectively. The intricate interplay of these peptide-dependent recognition processes results in the initiation or propagation of immune responses controlling, for example, infections and cancer in a subject, such as a human subject.

Vaccines have been designed based on the use of short synthetic peptides which mimic the exact epitope recognised by cytolytic CD8+ T lymphocytes when associated with the restricting MHC complex. This limits the applicability of the vaccine to individuals of the appropriate MHC haplotype. Since HLA alleles are extremely polymorphic, the practical approach to this type of vaccination has focused the efforts on those peptides presented by the most frequent HLA alleles. HLA-A2, and to a lesser extent other alleles such as -A1, -A3, -B7, -B35, are alleles generally relevant for individuals of Caucasian origin.

Despite HLA allele restriction, peptide vaccines offer considerable advantages such as absence of infectious
material capable of compromising live or attenuated vaccines. Furthermore, many pathogens can be difficult or impossible to culture by conventional methods. Peptide vaccine also offer the option to exclude deleterious sequences from full-length antigens, such as proteins, or other pathogen-derived molecules such as oncogenic compounds or compounds implicated in autoimmune diseases.

Peptides are easily characterised and analysed for purity using well-established analytical techniques such as liquid chromatography and mass spectrometry. This facilitates quality control and ultimately approval by the regulatory authorities.

The production of chemically defined peptides can be carried out economically on a large scale. Peptide preparations can be stored freeze-dried, which avoids the need to maintain a 'cold-chain' facility in storage, transport and distribution. There is no risk of reversion or formation of adverse reassortants that can lead to virulence, which is a potential problem associated with live attenuated vaccine preparations.

Peptide-based vaccines can be designed to include multiple determinants from several pathogens, or multiple epitopes from the same pathogen. The introduction of non-natural amino acids and peptide-like molecules into peptide-based vaccines allows the design of more drug-like compounds, which opens up avenues for vaccine delivery and rational drug design in vaccinology.

Despite the numerous advantages associated with the use of peptide vaccines, challenges in peptide vaccination strategies are, for example, the often low immunogenicity of the peptide, especially in the case of tumour antigens, the delivery of peptide epitopes to antigen presenting cells and premature peptide degradation by protease activity in the periphery or in APCs.
Modification of anchor amino acids by other naturally occurring amino acids may result in enhanced binding to the MHC and together with peptides in which TCR binding is altered, such peptides are designated altered peptide ligands or APLs. Substitutions in the TCR interacting region by naturally occurring amino acid, or heteroclitic analogues, may cause hyperstimulation of the CTL thereby providing a more potent immune response compared with the native epitope. Alternatively heteroclitic analogues may antagonise autoreactive CTLs, leading to immunosuppression, which can be exploited for the treatment of autoimmune disease and prevention of organ rejection following allogeneic transplantation.

Another strategy to improve the efficacy of peptide vaccines is the introduction into the peptide of non-naturally occurring amino acid residues, including incorporation of non-encoded alpha amino acids, photoreactive cross-linking of amino acids, beta-amino acids, backbone reduction, partial retro-inversion and incorporation of D-amino acids, N-terminal methylation and C-terminal amidation and pegylation. Synthetic engineering of peptide epitopes thus confers beneficial properties to the peptide vaccine such as improved MHC class I binding and TCR avidity, protease resistance, and oral bioavailability.

Immune restricted peptides, besides in vaccines, can also be used in immunosuppressive therapy and T cell antagonism. Currently, broad-spectrum drugs that generally suppress the immune system are used to reduce the risk of rejection after allogeneic organ transplantation (host versus graft reaction) or to lower the risk of Graft-versus-Host Disease after hematopoietic stem cell (bone marrow) transplantation.

CD8+ T cells have been implicated in mediating Graft-versus-Host Disease, but also early allograft
rejection, indicating an important role for MHC class I. Also the treatment of autoimmune diseases is based on immunosuppression. The selective knock-down of autoimmune or rejective responses is desirable and hitherto research has been focused on the design of modified versions of the natural pathogenic viral or self-antigenic peptides.

These altered peptide ligands (APLs) are epitopes in which one or multiple of the naturally occurring amino acid residues are replaced by another amino acid residue. They either block the MHC peptide binding groove, inhibiting binding to the TCR, or they antagonise the TCR, i.e. interaction with the TCR does take place, but without the onset of signalling.

Optimisation of immunogenic peptides is valuable for the generation of MHC multimers, which are widely used for epitope restricted T cell detection and isolation for adoptive T cell therapy.

Typically, a substantial proportion of T cell defined tumour-derived antigenic peptides are suboptimal for binding to HLA, with consequent fast dissociation from MHC and weak immunogenicity. Non-natural amino acid substitutions increase peptide binding to MHC resulting in highly stable complexes. It has been observed that the half-life of MHC/peptide complexes is directly correlated to immunogenicity. MHC multimers containing optimised tumour derived antigens, i.e. immunogenic peptides, aid in the isolation and subsequent expansion of, for example, tumour infiltrating lymphocytes.

Considering the above, it is an object of the present invention, amongst other objects, to provide immunogenic peptides with improved efficiency in, for example, vaccines, immunosuppressive therapy, adoptive T cell therapy and diagnostics.
Specifically, the present invention enables a new vaccination technology based on stable peptides that have the ability to induce T cell activation at very low epitope concentrations and/or at late timepoints after epitope binding to antigen-presenting cells, as an initial prevention against major health threats such as pandemic influenza. In addition, high burden diseases including cancer, such as melanoma, can be targeted with the present peptides.

Further, the present peptides enable inactivation of T cells by blocking the MHC-TCR interaction or by antagonising the T cell receptor.

Furthermore, the present peptides contribute to enhancing MHC multimer technology which is fundamental technique in monitoring infection and cancer, determining vaccination efficiencies and evaluating and isolating T cells for adoptive T cell therapy.

The above objects, amongst other objects, are met by immune restricted peptides as defined in the appended claim 1.

The term "immune restricted peptides", within the context of the present invention, designates modified peptides capable of eliciting, or modifying an immune response. The modification of the present peptides comprises the replacement, or substitution, of one ore more amino acids in a peptide, i.e. a peptide representing one or more immunogenic epitopes, with non-naturally occurring amino acids. The present immune restricted peptides can provide an increased immunogenicity as compared the original peptide or are capable to provide immunogenicity to original non-immunogenic peptides.

The term "non-naturally occurring amino acids" within the context of the present invention denotes amino acids which are not found in naturally occurring compounds.
such as proteins and peptides. Specifically, non-naturally occurring amino acids according to the present invention are not the L-amino acids: alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan and tyrosine.

Specifically, the above objects, amongst other objects, are met by immune restricted peptides, preferably HLA-A2 restricted peptides, according to the general formula (I):

\[ P_1 - P_2 - P_3 - P_4 - \left( P_m \right)_n - P_{c-2} - P_{c-1} - P_c \]

wherein:
- \( P_1 \) is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear aliphatic, aromatic or heteroaromatic substitution;
- \( P_2 \) is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear or branched substitution;
- \( P_3 \) is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear or branched substitution;
- \( P_4 \) is a naturally, or non-naturally, occurring amino acid comprising an N-alpha methyl substitution.
- \( P_m \) is a naturally occurring amino acid, \( n \) is an integer of 2 to 10, preferably 2 to 5, more preferably 3 or 4;
- \( P_{c-2} \) is a naturally, or non-naturally, occurring amino acid comprising a fluorinated aromatic substitution;
- \( P_{c-1} \) is a naturally, or non-naturally, occurring amino acid;
- \( P_c \) is a naturally, or non-naturally, occurring amino acid comprising unsaturated side chains and/or carboxyl isoesters;
under the condition that at least one of \( P_1, P_2, P_3, P_{c-2} \) and \( P_c \) is a non-naturally occurring amino acid.

The present peptides are based on chemically enhanced and/or stabilised variants of immunogenic or non-immunogenic peptides also designated as Epitopes'. Chemical enhancement and stabilisation of epitopes comprises the incorporation of non-naturally occurring amino acids. The present chemical enhancement and stabilisation of epitopes, or peptides, results, for example, in an improved proteolytic stability and/or enhanced HLA affinity, providing an enhanced immunogenicity and/or T cell antagonism as compared to the original, or non-modified, peptide.

The present invention preferably relates to HLA-A2 restricted epitopes, or HLA-A2 immune restricted peptides, with enhanced affinity for HLA-A2 comprising 8- to 16-, preferably 8- to 13-, more preferably of 9- or 10-mer peptides, based on naturally occurring HLA-A2 restricted epitopes in which at least one amino acid has been replaced by a non-natural modification thereof.

To generate the present immunogenic epitopes, the modifications, as defined above, can be introduced on amino acids \( P_1, P_2 \) and/or \( P_3 \) (counting from the N-terminus) and on the last \( (P_c) \) and second before last \( (P_{c-2}) \) amino acid. Amino acids between \( P_3 \) and the before last amino acid residue \( (P_{c-2}) \) are essential for T cell receptor activation.

\( P_{c-1} \) generally is any of the standard 20 naturally occurring side chains. Although substitution of this position provides improved binding to the HLA proteins, non-naturally occurring modifications on this position do not lead to activation of T cells. However, non-naturally occurring substitutions at \( P_{c-1} \) are beneficial for the development of T cell antagonists. Accordingly, an additional modification of the naturally occurring amino
acid at this position by a non-naturally occurring amino acid is contemplated within the context of the present invention.

According to a preferred embodiment, the present invention relates to immune restricted peptides, preferably HLA-A2 Immune restricted peptides, wherein at least two, preferably at least 3, more preferably at least 4, most preferably 5, of $P_1$, $P_2$, $P_3$, $P_4$, $P_{c-2}$ and $P_c$ are a non-naturally occurring amino acid.

Specifically preferred combinations of $P_1$, $P_2$, $P_3$, $P_4$, $P_{c-2}$ and $P_c$ are modification of $P_i$ in combination with $P_2$ and $P_c$, $P_i$ in combination with $P_2$, $P_{c-2}$ and $P_c$, $P_2$ in combination with $P_{c-2}$ and $P_c$, $P_2$ in combination with $P_c$, $P_2$ in combination with $P_{c-2}$, $P_i$ in combination with $P_2$, $P_i$ in combination with $P_{c-2}$ and $P_2$ in combination with $P_{c-2}$.

According to another preferred embodiment of the present invention:

$$P_4 \left[P_m\right]_{j=n}$$

is here defined as the residues that make up the bulk part of the interaction site between peptide and TCR, and is preferably part of an HLA-A2 restricted immunogenic epitope. An immunogenic epitope according to the present invention is an amino acid sequence capable of T cell activation.

Analogous, an HLA-A2 immunogenic epitope according to the present invention is an amino acid sequence capable of T cell activation through HLA-A2 presentation.

According to yet another preferred embodiment, the present invention relates to immune restricted peptides, preferably HLA-A2 immune restricted peptides, according to the general formula (II):

$$P_4 \left[P_m\right]_{j=n}$$
wherein:
- $R_1$ is methyl; $R_2$ is a substituted or non-substituted benzyl group and $R_3$ and $R_4$ are $H$;
- $R_1$ and $R_2$ are $H$; $R_3$ is $H$; $R_4$ is a fluorinated or non-fluorinated saturated linear aliphatic chain containing 2 to 6 carbon atoms with or without an oxygen atom or a sulfur atom within the chain, or an aromatic moiety including a substituted or non-substituted phenyl ring, a heteroaromatic moiety including substituted or non-substituted 2-, 3-, or 4-pyridine ring.
- $R_1$ and $R_2$ are $H$; $R_3$ is methyl; $R_4$ is an aromatic moiety or a substituted or non-substituted phenyl ring; or
- $R_1$ and $R_2$ are $H$; $R_3$ is $C\equiv$; $R_4$ is an aromatic moiety or a substituted or non-substituted phenyl ring.
- $R_6$ is a fluorinated or non-fluorinated saturated linear aliphatic chain containing 2 to 6 carbon atoms with or without an oxygen atom or a sulfur atom within the chain;
- $R_7$ is a fluorinated benzyl moiety;
- $R_8$ is an unsaturated carbon chain comprising of 2 to 3 carbon atoms and $R_9$ is carboxyl; or
- $R_8$ is a saturated or unsaturated linear or branched carbon chain containing 2 to 4 carbon atoms with or without an oxygen atom or a sulfur atom within the chain, or a terminal thiol group and $R_9$ is selected from the group consisting of carboxylate, tetrazole, boronate, phosphonate, N-acyl-S-alkyl-sulf onamide and hydroxamate;
- $R_{10}$ is $H$ or methyl
R\textsubscript{m} is a naturally occurring amino acid side chain.

According to the present invention, the present non-naturally occurring amino acid at positions \( P_1, P_2, P_3, P_{c-2} \) and \( P_c \) are preferably selected from the group consisting of TIC, CSME, OM-HS, NVA, NLE, BUTALA, PRG, PHG, SOME, 2-AOC, C\textsubscript{p}ALA, ALG, am-phg, 3-PYRA and 4-FPHE.

According to especially preferred embodiments of the present invention: \( P_{c-2} \) is 4-FPHE, \( P_1 \) is selected from the group consisting of am-phg, PHG, 3-PYRA and CSME, \( P_2 \) is selected from the group consisting of C\textsubscript{p}ALA, NLE, BUTALA, NVA and 2-AOC, \( P_3 \) is NLE, \( P_4 \) is an alpha-N-methylated amino acid residue containing a naturally occurring side-chain and/or \( P_c \) is selected from the group consisting of ALG, PRG, NLE, and OM-HS.

The preferred immunogenic epitopes according to the present invention as represented by

\[
P_4 \underbrace{\cdots}_n P_m
\]

are GFV, part of the HLA-A2 restricted influenza A matrix protein 1 (58-66) epitope, GIGI, part of the HLA-A2 restricted melanoma Mart-1 (26-35) epitope, or DFF, part of the HLA-A2 restricted melanoma TRP-2 (180-188) HLA-A2 epitope.

Considering the beneficial properties of the present immunogenic restricted, or modified, peptides according to the present invention, especially in the fields of vaccines, immunosuppressive therapy, adoptive T cell therapy and diagnostics, the present invention, according to another aspect, relates to a method for providing a immune restricted peptide, preferably an HLA-A2 restricted immunogenic peptide, comprising:
a) selecting an immunogenic peptide, preferably an HLA-A2 immunogenic peptide, represented by the formula

\[ P_1 - P_2 - P_3 - P_4 - \left[ P_m \right] - P_{c-2} - P_{c-1} - P_c \]

wherein \( P_1, P_2, P_3, P_4, P_m, P_{c-2}, P_{c-1} \) and \( P_c \) are naturally occurring amino acids;

b) replacing at least one of the naturally occurring amino acids at positions \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \) by a non-naturally occurring amino acid as defined above thereby providing a restricted immunogenic peptide, preferably an HLA-A2 restricted immunogenic peptide.

According to a preferred embodiment of the present method, the method comprises an additional step after step (a), but before step (b), comprising analysing the amino acid sequence of the immunogenic peptide using a computer algorithm providing a prediction of the at least one of the naturally occurring amino acids at positions \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \) to be replaced by the non-naturally occurring amino acid and the identification thereof.

According to another preferred embodiment of this aspect, the present invention relates to a method wherein step (b) comprises replacing at least two, preferably at least three, more preferably at least four, most preferably 5 of the naturally occurring amino acids at positions \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \).

Specifically preferred combinations of \( P_1, P_2, P_3, P_{c-2} \) and \( P_c \) are modification of \( P_1 \) in combination with \( P_2 \) and \( P_{c-2} \), \( P_1 \) in combination with \( P_2, P_{c-2} \) and \( P_c \), \( P_2 \) in combination with \( P_{c-2} \) and \( P_{c-2} \), \( P_1 \) in combination with \( P_{c-2} \), \( P_1 \) in combination with \( P_{c-2} \), \( P_2 \) in combination with \( P_{c-2} \), \( P_1 \) in combination with \( P_{c-2} \).
According to the present invention, the present non-naturally occurring amino acid at positions $P_1$, $P_2$, $P_3$, $P_{c-2}$ and $P_c$ are preferably selected from the group consisting of TIC, CSME, OM-HS, NVA, NLE, BUTALA, PRG, PHG, SOME, 2-AOC, $C_p$ALA, ALG, am-phg, 3-PYRA and 4-FPHE.

According to an especially preferred embodiment of the present invention: $P_{c-2}$ is 4-FPHE, $P_1$ is selected from the group consisting of am-phg, PHG, CSME and 3-PYRA, $P_2$ is selected from the group consisting of $C_p$ALA, NLE, BUTALA, NVA and 2-AOC, $P_3$ is NLE, $P_4$ is an alpha N-methylated amino acid residue containing naturally occurring side-chains and/or $P_c$ is selected from the group consisting of ALG, PRG, NLE, and OM-HS.

The present variant or modified peptides provide beneficial properties especially in the fields of vaccines, immunosuppressive therapy, adoptive T cell therapy and diagnostics. Accordingly, according to another aspect, the present invention relates to the use of the present immune restricted peptides in medicine.

Preferably, the present immune restricted peptides are in vaccines, in immunosuppressive therapy or T cell antagonism, diagnostic and/or in adoptive T cell therapy.

The present invention will be further detailed in the examples below outlining especially preferred embodiments of the present immune restricted peptides. In the examples, reference is made to figures wherein:

**Figure 1**: is a schematic representation of HLA binding peptides comprising non-naturally occurring amino acid modifications enhancing HLA binding affinity;

**Figure 2**: shows a representative example of a flow cytometry output image. T cells can be CD8 negative (lower left quadrant), CD8 positive (APC) but not MHC tetramer positive (streptavidin-PE), CD8 positive
(FITC), but not interferon-γ (APC) positive (upper left quadrant) or double positive (upper right quadrant);

**Figure 3**: shows the chemical structures of preferred non-naturally occurring amino acids, their IUPAC names and their abbreviations;

**Figure 4**: shows a schematic representation of a T cell activation time course assay. Interferon-γ production was determined at several time points.

**EXEMPLARY**

**Introduction**

In the examples below, optimisation of HLA A2 epitopes (**Figure 1**) is evaluated using the following techniques: HLA binding affinity of peptides is determined using an MHC exchange fluorescence polarisation assay. Binding of peptide MHC to T cells is assessed using MHC multimer technology. T cell activation by chemically enhanced epitopes is determined using an interferon-γ (IFNy) production assay. IFNy is a cytokine, predominantly produced upon T cell activation.

Both the IFNy and MHC tetramer assays are monitored using flow cytometry, in which fluorescently labelled cells can be detected (**Figure 2**). MHC-TCR interaction is visualised by phycoerythrin (PE) conjugated MHC-streptavidin tetramers and allophycocyanin (APC) conjugated anti-CD8 antibody capable of staining CD8+ T cells. Double positive cells are indicative of CD8+ T cells bound to peptide-MHC tetramers. Both the percentage of tetramer binding CD8+ T cells and the efficiency of tetramer staining per T cell, represented by the geometric mean (displayed as arbitrary fluorescence units) are taken into account.
IFNY production is visualised by intracellular staining using an APC conjugated anti-IFNY antibody, whereas the CD8+ T cell is stained with a fluorescein isothiocyanate (FITC) labelled anti-CD8 antibody. Both the percentage of IFNY producing T cells and the amount of IFNY produced per T cell (represented by the geometric mean) are taken into account.

In the examples below, T cell receptor exposed residues are left unchanged in order to maintain immunogenicity. The immunogenic activity of both high and low affinity epitopes has been enhanced with relative ease. An increase in HLA binding affinity up to a factor 1000 has been achieved. Epitopes enhanced by the invented technology presented here showed increased T cell stimulatory activity, as determined by IFNY production, compared to native epitopes. The chemical structures of the non-naturally occurring amino acids used below are presented in Figure 3.

**Material and methods**

HLA binding affinity was determined by a fluorescence polarization (FP) assay based on UV mediated MHC peptide exchange. In short, purified soluble MHC class I molecules (HLA-A0201) loaded with a UV-labile peptide KILGFVFJV, in which J is photocleavable 3-amino-3-(2-nitrophenyl)propionic acid, (5.3 µM stock) are used for this assay. MHC molecules are diluted in phosphate buffer saline containing 0.5 mg/ml bovine gamma globulines (referred to as PBS/BGG) to a final concentration of 0.75 µM and pipetted into a 96 well microplate.

The HLA-A2 restricted hepatitis B virus epitope, FLPSDCFPSSV, fluorescently labelled with tetramethylrhodamine (TAMRA) covalently bound to the cysteine residue, is used as the tracer. This tracer peptide is diluted in PBS/BGG to a
concentration of 6 nM and manually pipetted into a 96 well microplate. The peptides of interest are diluted in DMSO to a concentration of 125 µM and pipetted into a 96 well microplate. A Hamilton high throughput liquid handling robot is then used to combine the components from the three 96 well microplates into a black nonbinding surface 384 well microplate so that each peptide can be measured in triplicate for the fluorescence polarization assay. Once all the components are in the 384 well microplate (30 µL per well of 0.5 µM MHC, 1 nM tracer and 5 µM peptide), the plate is spun down to mix all the components and to remove any air bubbles.

Competition between the tracer peptide and the peptides of interest starts when the 384 well microplate is placed under a UV-lamp (> 350 nm) for 30 minutes at 4 °C to cleave the UV-labile peptide.

All scores represent the percentage inhibition of the FP of the fluorescent tracer peptide. IC50 values are represented as fold increase towards the index peptide, which is set to an arbitrary value of 1.

Peptide/MHC (p/MHC) binding to the TCR was analysed by Fluorescence Assisted Cell Sorting (FACS) on a BD FACSCalibur machine, where 20,000 to 30,000 events were counted per sample. In short, enhanced and control peptides were pipetted in DMSO to a final concentration of 500 µM in a 96 well microplate. Biotinylated MHC monomers (2.45 mg/ml stock) were then diluted in PBS to 25 µg/ml and dispensed with non-binding surface pipette tips, 27 µL/per well in a 96 well microplate. 3 µL of the peptide plate was added to the MHC monomer plate and UV-irradiated for 30 minutes. The plates were then left at RT for another 30 minutes.

Subsequently, the plates were centrifuged for 5 minutes at 3300 RCF to remove disintegrated MHC molecules and 20 µL supernatant was transferred to a new 96 well
20 µl of PBS-diluted streptavidin-R-phycoerythrin conjugate (27 µg/ml) was added to the peptide-MHC plate in 4 x 15 minute intervals. The intervals are necessary to saturate the streptavidin molecules with the biotinylated MHC molecules so that the maximum amount of fully loaded tetramers is achieved.

In the same time, 100,000 T cells per well were plated out by a Thermo Scientific wellmate cell dispenser in a 384 well microplate. A Hamilton high throughput liquid handling robot was used to add 2 µl of p/MHC-tetramer in triplicate from the 96 well microplate into the cell-filled 384 well microplate. This plate was then incubated for 15 minutes at 37°C. Then 4 µl of allophycocyanin conjugated anti-CD8 antibody (8 x diluted in PBS) was added to each well and incubated for 20 minutes on ice. Subsequently, after two wash steps with PBS, the wells were filled with PBS containing PI to distinguish between live and dead T cells in the FACS analysis. Data were analysed using FCS Express 2 by De Novo software and Microsoft Excel.

T cell activation assays (Figure 4) based on IFNγ production were carried out using a BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiPlug™. FACS was employed to obtain results. As antigen presenting cells, T2 cells or JY cells were used, pulsed with different concentrations of our peptides for the duration of one hour at 37°C.

T2 cells were used as antigen presenting platform and were cultured in RPMI medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin. T cells were grown in RPMI/AIM-V medium (50:50), supplemented with 10% human serum, penicillin and streptomycin, interleukin-2 and glutamax. 50,000 T2 cells were plated out per well and peptides were added to a 1 µM final concentration. T2 cells and peptides were incubated at 37 °C for 1H after which
50,000 T cells in 50 µl medium were added to the T2 plate. 100 µl of RPMI medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin was added to the wells and the plate was spun at 1500 rpm for 3 minutes.

The supernatant was discarded and 100 µl of GolgiPlug (1 µl/ml) in RPMI medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin was added to the cells. For the positive control, 2 µl of phorbol 12-myristate 13-acetate (PMA) and 2 µl of ionomycin diluted in GolgiPlug medium was added to the positive control cells.

The plate was spun at 700 rpm for 2 minutes and incubated for 4 hours at 37 °C.

After incubation the plate was spun at 1300 rpm for 3 minutes and the supernatant was discarded. The cells were resuspended in 50 µl of FACS buffer with FITC labelled anti-CD8 antibody (20 µl/ml) and left to stain for 15 minutes in the dark at room temperature.

After staining the plate was spun at 1300 rpm for 3 minutes, and two wash steps were performed in which the cells are washed with 300 µl of FACS buffer. The cells were resuspended in 100 µl of Cytofix/Cytoperm solution and incubated on ice for 20 minutes. The plate was spun at 1300 rpm for 3 minutes and the supernatant was discarded and replaced by 250 µl of Permwash; this step was repeated. The cells were resuspended in 50 µl of Permwash with APC conjugated anti-IFNy antibody. PermWash buffer was used for the dilution of the APC conjugated anti-IFNy antibody, rather than a standard buffer, in order to maintain cells in a permeabilised state for the intracellular staining. The plate was incubated on ice for 30 minutes. The plate was spun at 1300 rpm for 3 minutes and the supernatant was discarded and replaced by 250 µl of Permwash; this step was repeated.
After the final wash step, the supernatant was discarded and the cells were resuspended in FACS buffer. Cells were then transferred from the plate into FACS-tubes and the samples were analysed by FACS. Data were analysed using FCS Express 2 by De Novo software and Microsoft Excel.

**Example 1:** GILGFVFTL - Influenza A, Matrix Protein 1, residues 58-66

The Influenza A Matrix 1 epitope is a highly conserved epitope amongst Influenza A variants and binds strongly to HLA-A2. This epitope serves as a model for stringent selection of unnatural amino acid modifications. Modifications and evaluation of HLA binding and T cell reactivity are summarised in **Table 1**. Replacements found to enhance the HLA affinity of this epitope, were also found to be beneficial to HLA binding of other epitopes (see examples 2 and 3 below).
Table 1: HLA affinity and T cell recognition of, and T cell activation by optimized Influenza A, Matrix Protein 1 (58-66) analogues.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>% inhibition at T=4H and T=24H</th>
<th>IC₅₀ Ratio</th>
<th>% Positive TCR recognition</th>
<th>Geometric mean IFN in arbitrary fluorescence units</th>
<th>% CD8+ T cells producing IFN-γ</th>
<th>Geometric mean IFN-γ in arbitrary fluorescence units</th>
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<td>1</td>
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<td>[am-phg] LGFV [4-FPHE] TL</td>
<td>96 96 5 3.26 208 2.8 243</td>
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<td>3</td>
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<td>77 77 4 3.58 328 2.7 238</td>
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<td>4</td>
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<td>83 84 3 3.20 217 2.6 266</td>
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<tr>
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<tr>
<td>11</td>
<td>GILGFVFTL</td>
<td>81 78 1 3.80 219 2.6 237</td>
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</tr>
<tr>
<td>12</td>
<td>FLPSDFFPSV</td>
<td>89 87 3 0.10 100 0.0 106</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
The columns listed under Sequence' numbering 1 to 9 indicate the amino acid residue present on the designated position in the epitope.

CD8+ T cells were obtained from Influenza A positive donors and were sorted using tetramers containing HLA A2.1::GILGFVFTL. The hepatitis B viral epitope FLPSDFFPSSV (entry 12) was used as a negative control peptide in the TCR binding and IFNγ production assays. This natural epitope is known for its very high affinity for HLA-A2.1.

 FP 4H and 24H represent percentage inhibition of tracer peptide binding by 5 µM competitor peptide at 4 hours and 24 hours incubation, respectively. High inhibition values maintained over 24 hours indicate a low off-rate of the peptide and consequently long lived p/MHC complexes.

 IC50 values were determined using the MHC exchange FP assay and IC50 ratios represent IC50 values determined using the FP MHC exchange assay normalised to the native index peptide (entry 11).

 %TCR shows the percentage of CD8+ T cells that are stained by the indicated p/MHC-tetramers. GeoTCR represents T cell staining efficiency.

 The last two columns represent IFNγ production by stimulated T cells. %IFN indicates the percentage of T cells that are both CD8+ and produce IFNγ, whereas GeoIFN indicates the amount of IFNγ per T cell. 1.5 µg peptide was added per well to load antigen presenting cells and IFNγ production at time point 4H after adding T cells to the antigen presenting cells was measured.

 In short, all substitutions introduced in Influenza A matrix 1 epitope (58-66) lead to enhanced HLA-A2 affinity, and similar or improved T cell activation efficiency (entries 2 to 10) compared to the native epitope.
Example 2: EAAGIGILTV - Melanoma, Mart-1, residues 26-35

The melanoma epitope EAAGIGILTV has low HLA affinity. When it became clear that defined anchor residues exist for all specific HLA types, replacement of alanine on P2 by a leucine was used to create an altered peptide ligand with greater MHC affinity, while maintaining T cell activation of lymphocytes that respond to native epitope EAAGIGILTV. The A to L mutation enhances MHC affinity, but not to the extent that is shown below by introduction of unnatural substitutions. Modifications and evaluation of HLA binding and T cell reactivity are summarised in Table 2.
### Table 2: HLA affinity, T cell recognition and T cell activation by optimized Melanoma, Mart-1 (26-35) analogues

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>% inhibition at T=4H and T=24H</th>
<th>% Positive TCR recognition</th>
<th>Geometric mean in arbitrary fluorescence units</th>
<th>% CD8+ T cells producing IFN</th>
<th>Geometric mean IFN in peptide concentrations in picomolar</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>[CSME] [2-AOC] AGIGILT</td>
<td>72 71 12.2 11</td>
<td>212</td>
<td>8 8 8 6 2 1</td>
<td>177 179 130 101 76 73</td>
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</tr>
<tr>
<td>2</td>
<td>[am-phg] [NVA] AGIGILT [ALG]</td>
<td>58 63 2.5 9</td>
<td>321</td>
<td>9 8 6 3 1 1</td>
<td>106 106 82 66 63 72</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>[am-phg] [NVA] AGIGILT [PRG]</td>
<td>67 64 8.3 11</td>
<td>314</td>
<td>6 6 5 3 2 1</td>
<td>139 152 109 82 72 87</td>
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</tr>
<tr>
<td>4</td>
<td>[am-phg] LAGIGILT [PRG]</td>
<td>68 65 7.2 10</td>
<td>334</td>
<td>8 6 5 2 1 1</td>
<td>93 94 74 62 70 63</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>[am-phg] [2-AOC] AGIGILT [PRG]</td>
<td>80 77 24.3 10</td>
<td>283</td>
<td>5 5 5 2 1 1</td>
<td>162 161 123 82 83 76</td>
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</tr>
<tr>
<td>6</td>
<td>[4-FPHE] 4AGIGI [4-FPHE] TV</td>
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<td>ND</td>
<td>ND ND ND ND ND ND ND ND ND ND</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>[am-phg] [NVA] AGIGILT [4-FPHE] TV</td>
<td>65 63 10.1 9</td>
<td>173</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>[am-phg] [NLE] AGIGILT [PRG]</td>
<td>73 72 6.6 11</td>
<td>301</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>[am-phg] 4AGIGI [4-FPHE] TV</td>
<td>62 59 2.1 ND</td>
<td>ND</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>[CSME] [2-AOC] AGIGILT [PRG]</td>
<td>72 70 14.8 10</td>
<td>264</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>[CSME] [NLE] AGIGILT TV</td>
<td>62 60 3.2 11</td>
<td>223</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>[CSME] [NVA] AGIGILT</td>
<td>82 77 3.2 11</td>
<td>224</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>[CSME] [NVA] AGIGILT [PRG]</td>
<td>63 60 2.8 11</td>
<td>200</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>[PHG] [NVA] AGIGILT [ALG]</td>
<td>65 64 18.9 9</td>
<td>194</td>
<td>ND ND ND ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In the columns listed under Sequence' numbering 1 to 10 indicates the amino acid present on the designated position in the epitope. HLA-A2::Mart-1 (26-35) reactive T cells were obtained either by transduction of CD8+ T cells with a viral vector containing a monoclonal TCR for EAAGIGILTV or were isolated from melanoma patients and sorted using MHC tetramers containing HLA-A2.1::ELAGIGLTV. FP 4H and 24H represent percentage inhibition of tracer peptide binding by 5 μM competitor peptide at 4 hours and 24 hours incubation, respectively. High inhibition values maintained over 24 hours indicate a low off rate of the peptide and consequently long lived p/MHC complexes.

IC_{50} values were determined using the MHC exchange FP assay and were normalised to the well known A2L altered peptide ligand (ELAGIGILTV), represented as IC_{50} ratios. %TCR and GeoTCR are to be interpreted as in Table 1. The data shown were obtained using T cells transduced with a viral vector containing a monoclonal TCR for EAAGIGILTV.

%IFN and GeoIFN are to be interpreted as in Table 1. Peptide concentrations ranged from 100 pM to 0.005 pM.

The data shown were obtained using FACS-sorted T cells derived from a single patient, thus containing EAAGIGILTV reactive TCRs. The wild type epitope EAAGIGILTV was not included as a control because previous experiments indicated that at the concentrations used in this assay, this peptide did not induce measurable IFNγ expression.

The introduction of multiple nonnatural amino acid residues yielded peptides with up to a factor 24 higher HLA-A2 binding affinity (entry 5) according to the IC_{50} data compared to the reported A2L modification. Compared to the
original wild type epitope a 300-400 fold increase in HLA affinity is observed.

TCR binding data show that most of the optimised peptides display similar or enhanced T cell staining efficiency as compared to native or A2L modified epitopes. It is also observed that [4-FPHE] on P ε-2 increases HLA affinity but not T cell activation. Whereas in the Influenza epitope [4-FPHE] replaces a phenylalanine which it closely resembles, here it replaces a leucine on a site exposed to the TCR. Apparently, interaction between MHC loaded with this peptide analogue and the TCR is hampered. Consequently, the introduction of [4-FPHE] on P ε-2 does not constitute a general improvement of immunogenicity, but is dependent on the particular epitope-TCR combination.

Titration of peptide analogues in the IFNy assay revealed a tenfold higher T cell stimulatory activity of [CSME] [2-AOC] AGIGILTV (entry 1) compared to control epitope ELAGIGILTV (entry 29).

After determination of optimal peptide concentrations in the titration assay, an experiment was set up in which interferon-γ production by T cells upon stimulation by APC's (here T2 cells) displaying modified epitopes was monitored over time as is schematically shown in Figure 4. The standard 4 hour timepoint was also taken along. For the 24h and 48h time points free peptide was washed away at the times indicated in Figure 4 to assess how long MHC complexes presenting the modified epitopes are present at the cell surface of the APC’s at a concentration sufficient to induce T cell activation.

The experiment involved the same modified peptides as used earlier in the titration assay and was carried out using peptide concentrations of 50 pM (Table 3) and 0.5 pM (Table 4), respectively.
**Table 3:** HLA affinity and T cell activation time course by optimized Melanoma, Mart-1 (26-35) analogues added at 50 pM

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>% inhibition at T=4H and T=24H</th>
<th>IC₅₀ Ratio</th>
<th>% CD8+ T cells producing IFN-γ</th>
<th>Geometric mean IFN-γ in arbitrary fluorescence units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FP at 4h</td>
<td>FP at 24h</td>
<td>OH 2H 4H 24H 48H</td>
<td>OH 2H 4H 24H 48H</td>
</tr>
<tr>
<td>1</td>
<td>[am-phg] [NVA] AGIGILT [ALG]</td>
<td>58 63</td>
<td>2</td>
<td>0 28 33 29 20</td>
<td>109 247 715 400 280</td>
</tr>
<tr>
<td>2</td>
<td>[am-phg] LAGIGILT [PRG]</td>
<td>68 65</td>
<td>7</td>
<td>0 22 31 28 18</td>
<td>105 249 480 342 215</td>
</tr>
<tr>
<td>3</td>
<td>[am-phg] [NVA] AGIGILT [PRG]</td>
<td>67 64</td>
<td>8</td>
<td>0 28 32 28 18</td>
<td>110 252 550 344 222</td>
</tr>
<tr>
<td></td>
<td>[am-phg] [2-AOC] AGIGILT [PRG]</td>
<td>80 77</td>
<td>24</td>
<td>0 27 30 16 1</td>
<td>109 258 592 187 86</td>
</tr>
<tr>
<td>4</td>
<td>[CSME] [2-AOC] AGIGILTV</td>
<td>72 71</td>
<td>12</td>
<td>0 28 31 27 20</td>
<td>111 249 563 397 288</td>
</tr>
<tr>
<td>5</td>
<td>ELAGIGILTV</td>
<td>62 58</td>
<td>1</td>
<td>0 26 31 16 2</td>
<td>104 243 498 173 97</td>
</tr>
</tbody>
</table>
The OH time point represents basal IFNγ levels of T cells in a resting state. After 2 hour incubation with peptide-MHC presenting cells, IFNγ levels have risen considerably, reaching maximum levels, as measured here at 4 hours and gradually declining at longer time points. While up to 4 hours no significant differences between index and modified peptides are apparent, at time points 24 and 48H distinct differences are found. With the exception of entry 4 all modified peptides display the ability to activate T cell for a longer duration (up to the 48 h measured) than the index peptide.
Table 4: HLA affinity and T cell activation time course of optimized Melanoma, Mart-1 (26-35) analogues added at 0.5 pM

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>% inhibition at T=4H and T=24H</th>
<th>IC₅₀ Ratio</th>
<th>% CD8+ T cells producing IFN-γ</th>
<th>Geometric mean IFN-γ in arbitrary fluorescence units</th>
</tr>
</thead>
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<tr>
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<td>FP 4h</td>
<td>FP 24h</td>
<td>OH</td>
<td>2H</td>
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<td>63</td>
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<td>0</td>
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<tr>
<td>2</td>
<td>[am-phg] LAGIGILT [PRG]</td>
<td>68</td>
<td>65</td>
<td>7</td>
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<tr>
<td>3</td>
<td>[am-phg] [NVA] AGIGILT [PRG]</td>
<td>67</td>
<td>64</td>
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<tr>
<td>4</td>
<td>[am-phg] [2-AOC] AGIGILT [PRG]</td>
<td>80</td>
<td>77</td>
<td>24</td>
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<tr>
<td>5</td>
<td>[CSME] [2-AOC] AGIGILTV</td>
<td>72</td>
<td>71</td>
<td>12</td>
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<tr>
<td>6</td>
<td>ELAGIGILTV</td>
<td>62</td>
<td>58</td>
<td>1</td>
<td>0</td>
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</tbody>
</table>
Also incubation with 0.5 pM peptide shows that all modified peptides, with the exception of entry 4, are able to induce IFNγ production in responding T cells for a longer period of time than the index peptide. Moreover, as also seen with 50 pM peptide incubation, the duration of T cell activation by modified peptides is extended up to 48 hours. In contrast, at the 24 hour time point index peptide and entry 4 induce IFNγ production only just measurable above background level.

Example 3 SVYDFFVWL - Melanoma, TRP-2, residues 180-188

This epitope stems from tyrosinase-related protein 2 (TRP-2), an enzyme expressed in most melanoma cancers. It has a moderate affinity for HLA-A21 making it suitable for binding enhancement. Several modified epitopes were designed and binding data as well as T cell activation data on 19 of these are plotted in Table 5.

Also for this epitope, an IFNγ expression time-course experiment was performed. The two distinguishing time points 24H and 48H were taken along at peptide concentrations of $5 \times 10^3$ pM, 50 pM and 0.5 pM. The $5 \times 10^3$ pM concentration was included because this specific T cell receptor was found to require higher peptide levels to reach the dynamic measuring range than for example the EAAGIGILTV reactive TCR discussed in Example 2.
### Table 5: HLA affinity and T cell activation (after 24 or 48 hours and at multiple peptide concentrations) by optimized Melanoma, Trp-2 (180-188) analogues

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>% inhibition at T=4H and T=24H</th>
<th>% CD8+ T cells producing IFN-γ peptide concentrations in picomolar</th>
<th>Geometric mean IFN-γ in arbitrary fluorescence units peptide concentrations in picomolar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24H</td>
<td>48H</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FP 4h</td>
<td>FP 24h</td>
</tr>
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<td>[am-phg] [NVA] YDFFVWL</td>
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<td>77</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>[CSME] [2-AOC] YDFFVWL</td>
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<td>76</td>
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<tr>
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<td>[PHG] [NVA] YDFFVWL</td>
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<td>76</td>
<td>29</td>
</tr>
<tr>
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<td>75</td>
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<td>5</td>
<td>[PHG] [CpALA] YDFFVWL</td>
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<td>75</td>
<td>30</td>
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<td>[PHG] [NVA] YDFFVW [PRG]</td>
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<tr>
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<td>[ALG]</td>
</tr>
<tr>
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<td>[2-AOC]</td>
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<td>[PRG]</td>
</tr>
<tr>
<td>20</td>
<td>SVYDFVWL</td>
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</tbody>
</table>
The results support the findings obtained for the Mart-1 ELAGIGILTV epitope. When enhanced by incorporating unnatural residues, the derivatives gain the ability to induce IFN\(\gamma\) expression for a longer period of time. Already at timepoint 24H a difference is observed at the 5-10\(^3\) pM concentration when comparing the natural epitope to the better performing derivatives. Even more so this same effect is apparent after 48H.

At 50 pM and 24H the natural epitope induced IFN\(\gamma\) expression has fallen to background level, whereas the enhanced epitopes still show significant expression of this cytokine. At the same concentration but after 48H, only entries 1, 2 and 6 still show IFN\(\gamma\) expression significantly above background level. For the epitope-TCR combination studied here, incubation with 0.5 pM peptide does not lead to T cell activation in all cases.

**Conclusion**

In summary, the present examples show that the unnatural peptide analogues, containing non-naturally occurring amino acids, display stronger MHC binding and show stronger and prolonged capacity to induce T cell activation at concentrations lower than required for their natural counterparts.
CLAIMS

1. Immune restricted peptide, preferably an HLA-A2 immune restricted peptide, according to the general formula (I):

\[ P_1 - P_2 - P_3 - P_4 - P_{m-1} - P_{c-2} - P_{c-1} - P_c \]

wherein:
- \( P_1 \) is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear aliphatic, aromatic or heteroaromatic substitution;
- \( P_2 \) is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear or branched substitution;
- \( P_3 \) is a naturally, or non-naturally, occurring amino acid comprising a hydrophobic linear or branched substitution;
- \( P_4 \) is a naturally, or non-naturally, occurring amino acid comprising a methylated alpha nitrogen atom;
- \( P_{m-1} \) is a naturally occurring amino acid, \( n \) is an integer of 1 to 9, preferably 1 to 4, more preferably 2 or 3;
- \( P_{c-2} \) is a naturally, or non-naturally, occurring amino acid comprising a fluorinated aromatic substitution;
- \( P_{c-1} \) is a naturally, or non-naturally, occurring amino acid;
- \( P_c \) is a naturally, or non-naturally, occurring amino acid comprising unsaturated side chains and/or carboxyl isoesters;

under the condition that at least one of \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \) is a non-naturally occurring amino acid.
2. Immune restricted peptide according to claim 1, wherein at least two, preferably at least 3, more preferably at least 4, most preferably 5, of \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \) are a non-naturally occurring amino acid.

3. Immune restricted peptide according to claim 1 or claim 2, wherein:

\[
P_4 \quad \left[ P_m \right]_n
\]

is here defined as the residues that make up the bulk part of the interaction site between peptide and TCR, and is preferably a fragment of an HLA-A2 restricted immunogenic epitope.

4. Immune restricted peptide according to any of the claims 1 to 3 according to the general formula (II):

\[
\text{R}
\]

wherein:
- \( R_1 \) is methyl; \( R_2 \) is a substituted or non-substituted benzyl group and \( R_3 \) and \( R_4 \) are H;
- \( R_1 \) and \( R_2 \) are H; \( R_3 \) is H; \( R_4 \) is a fluorinated or non-fluorinated saturated linear aliphatic chain containing 2 to 6 carbon atoms with or without an oxygen atom or a sulfur atom within the chain, or an aromatic moiety including a substituted or non-substituted phenyl ring, a heteroaromatic moiety including substituted or non-substituted 2-, or 3-, or 4-pyridine ring.
- $R_1$ and $R_2$ are $H$; $R_3$ is methyl; $R_4$ is an aromatic moiety or a substituted or non-substituted phenyl ring; or
- $R_1$ and $R_2$ are $H$; $R_3$ is CH$_3$; $R_4$ is an aromatic moiety or a substituted or non-substituted phenyl ring.

5. $R_6$ is a fluorinated or non-fluorinated saturated linear aliphatic chain containing 2 to 6 carbon atoms with or without an oxygen atom or a sulfur atom within the chain;
- $R_7$ is a fluorinated benzyl moiety;
- $R_8$ is an unsaturated carbon chain comprising of 2 to 3 carbon atoms and $R_9$ is carboxyl; or
- $R_8$ is a saturated or unsaturated linear or branched carbon chain containing 2 to 4 carbon atoms with or without an oxygen atom or a sulphur atom within the chain, or a terminal thiol group and $R_9$ is selected from the group consisting of carboxylate, tetrazole, boronate, phosphonate, $N$-acyl-$S$-alkyl-sulfonamide and hydroxamate;
- $R_{10}$ is $H$ or methyl;
- $R_m$ is a naturally occurring amino acid side chain.

5. Immune restricted peptide according to any of the claims 1 to 4, wherein the non-naturally occurring amino acid is selected from the group consisting of TIC, CSME, O$_2$-HS, NVA, NLE, BUTALA, PRG, PHG, SOME, 2-AOC, CpALA, ALG, am-phg, 3-PYRA and 4-FPHE.

6. Immune restricted peptide according to any of the claims 1 to 5, wherein $P_{12}$ is 4-FPHE.

7. Immune restricted peptide according to any of the claims 1 to 6, wherein $P_1$ is selected from the group consisting of am-phg, PHG, 3-PYRA and CSME.
8. Immune restricted peptide according to any of the claims 1 to 7, wherein $P_2$ is selected from the group consisting of C_pALA, NLE, BUTALA, NVA and 2-AOC.

9. Immune restricted peptide according to any of the claims 1 to 8, wherein $P_3$ is NLE.

10. Immune restricted peptide according to any of the claims 1 to 9, wherein $P_4$ is an alpha N-methylated amino acid.

11. Immune restricted peptide according to any of the claims 1 to 10, wherein $P_C$ is selected from the group consisting of ALG, PRG, NLE, CSME and OM-HS.

12. Immune restricted peptide according to any of the claims 1 to 11, wherein

$$P_4 \underbrace{P_m}_{n}$$

is selected from the group GFV, GIGI or DFF.

13. Method for providing an immune restricted peptide, preferably an HLA-A2 immune restricted peptide, comprising:

a) selecting an immunogenic peptide, preferably an HLA-A2 immunogenic peptide, represented by the formula

$$P_1 \underbrace{P_2}_{P_3} P_4 \underbrace{P_m}_{P_c} \underbrace{P_{c-1} P_{c-1}}_{P_C}$$
wherein \( P_1, P_2, P_3, P_4, P_m, P_{c-2}, P_{c-1}, \) and \( P_c \) are naturally occurring amino acids;

b) replacing at least one of the naturally occurring amino acids at positions \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \) by a non-naturally occurring amino acid as defined in any of the claims 1 to 12 thereby providing an immune restricted peptide, preferably an HLA-A2 restricted peptide.

14. Method according to claim 13, further comprising after step (a), but before step (b), analysing the amino acid sequence of the immunogenic peptide using a computer algorithm providing a prediction of the at least one of the naturally occurring amino acids at positions \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \) to be replaced by the non-naturally occurring amino acid and the identification thereof.

15. Method according to claim 13 or claim 14, wherein step (b) comprises replacing at least two, preferably at least three, more preferably at least four, most preferably 5 of the naturally occurring amino acids at positions \( P_1, P_2, P_3, P_4, P_{c-2} \) and \( P_c \).

16. Method according to any of the claims 13 to 15, wherein the non-naturally occurring amino acid is selected from the group consisting of TIC, CSME, OM-HS, NVA, NLE, BUTALA, PRG, PHG, SOME, 2-AOC, CpALA, ALG, am-phg, 3-PYRA and 4-FPHE.

17. Method according to any of the claims 13 to 16, wherein \( P_{c-2} \) is 4-FPHE.
18. Method according to any of the claims 13 to 17, wherein P₁ is selected from the group consisting of am-phg, PHG, 3-PYRA and CSME.

19. Method according to any of the claims 13 to 18, wherein P₂ is selected from the group consisting of CPALA, NLE, BUTALA, NVA and 2-AOC.

20. Method according to any of the claims 13 to 19, wherein P₃ is NLE.

21. Method according to any of the claims 13 to 20, wherein P₄ is an alpha N-methylated amino acid containing a naturally occurring side chain.

22. Method according to any of the claims 13 to 21, wherein P₅ is selected from the group consisting of ALG, PRG, NLE, and OM-HS.

23. Immune restricted peptide according to any of the claims 1 to 12 for use in medicine.

24. Immune restricted peptide according to any of the claims 1 to 12 for use as a vaccine.

25. Immune restricted peptide according to any of the claims 1 to 12 for use in immunosuppressive therapy or T cell antagonism.

26. Immune restricted peptide according to any of the claims 1 to 12 for use in adoptive T cell therapy.
27. Immune restricted peptide according to any of the claims 1 to 12 for use in diagnostics.
FIGURE 2

% Positive T cells

GeoMean

$2.8\times 10^3$

$10^2$

$10^1$

$10^0$

$10^{-1}$

$10^{-2}$

$10^{-3}$

Streptavidin-PE (tetramer binding)

OR APC (IFN-γ production)

OR CD8+ FITC (IFN-γ production)

CD8+ APC (tetramer binding)
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<tr>
<th>Compound</th>
<th>Structure</th>
<th>Description</th>
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<tr>
<td>CpALA</td>
<td><img src="image1" alt="CpALA structure" /></td>
<td>(S)-2-amino-3-cyclopropylpropanoic acid</td>
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<tr>
<td>PRG</td>
<td><img src="image2" alt="PRG structure" /></td>
<td>(S)-2-amino-4-propionic acid</td>
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<tr>
<td>SOME</td>
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<td>(S)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid</td>
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<td>BUTALA</td>
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<td>(S)-2-amino-4,4-dimethylpentanoic acid</td>
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<tr>
<td>PHG</td>
<td><img src="image5" alt="PHG structure" /></td>
<td>(S)-2-amino-2-phenylpentanoic acid</td>
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<tr>
<td>TTC</td>
<td><img src="image6" alt="TTC structure" /></td>
<td>(S)-2-amino-5-methoxypentanoic acid</td>
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<td>2-AOC</td>
<td><img src="image7" alt="2-AOC structure" /></td>
<td>(S)-2-amino-2-octanoic acid</td>
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<tr>
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<td>(S)-2-amino-5-methoxypentanoic acid</td>
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<td>NVFA</td>
<td><img src="image9" alt="NVFA structure" /></td>
<td>(S)-2-amino-4-pentanoic acid</td>
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<tr>
<td>am-phg</td>
<td><img src="image10" alt="am-phg structure" /></td>
<td>(S)-2-amino-2-phenylpropanoic acid</td>
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<tr>
<td>4-FPHE</td>
<td><img src="image11" alt="4-FPHE structure" /></td>
<td>(S)-2-amino-3-(4-fluorophenyl)propionic acid</td>
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<tr>
<td>CSME</td>
<td><img src="image12" alt="CSME structure" /></td>
<td>(S)-2-amino-3-(methylthio)propionic acid</td>
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<td>3-PYRA</td>
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<td>(S)-2-amino-3-(pyridin-3-yl)propionic acid</td>
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/11 C07K14/285 C07K14/705 A61K38/04

ADJ.

According to International Patent Classification (IPC) onto both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>X</td>
<td>B RODENKO ET AL.: “Cls ass I major hi stocompati bility comple xes loaded by a peri odate tri gger”</td>
<td>1-4, 12-15, 23-27</td>
</tr>
<tr>
<td>X</td>
<td>P H N CELI E ET AL.: “UV-i nduced ligand exchange in MHC class I protei n crystal s”</td>
<td>1-4, 12-15</td>
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X Further documents are listed in the continuation of Box C. ☑

☐ See patent family annex.

* Special categories of cited documents:

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Z document member of the same patent family

Date of the actual completion of the international search

7 September 2011

Date of mailing of the international search report

16/09/2011

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Authorized officer

Masturzo, Pietro
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