(19) **DANMARK**

(10) **DK/EP 2275441 T3**

(12) **Oversættelse af europæisk patentskrift**

Patent- og
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(54) Benævnelse: **Høj-affine HIV-T-celle-receptorer**

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# DESCRIPTION

[0001] The present invention relates to T-cell receptors (TCRs) having the property of binding to HIV Gag polypeptide-derived SLYNTVATL-HLA-A*0201. The TCRs comprise at least one TCR α chain variable domain and/or at least one TCR β chain variable domain and have a $K_D$ for the said SLYNTVATL-HLA-A*0201 complex of less than or equal to 1µM and/or has an off-rate $(k_{off})$ for the SLYNTVATL-HLA-A*0201 complex of $1x10^{-3}$ $S^{-1}$ or slower.

## Background to the Invention

[0002] The Human Immuno-deficiency Virus (HIV) is the causative agent of Acquired Immuno-deficiency Disease Syndrome (AIDS). The virus is an enveloped retrovirus belonging to the lentivirus group. The SLYNTVATL (SEQ ID NO: 16) peptide is derived from the g17 gene product of the Gag gene, one of nine genes which make up the Human Immuno-deficiency Virus-1 (HIV-1) The peptide is loaded by HLA-A*0201 and presented on the surface of HIV infected cells. Therefore, the SLYNTVATL-HLA-A2*0201 complex provides an HIV marker that TCRs can target, for example for the purpose of delivering cytotoxic or immuno-stimulatory agents to the infected cells. However, for that purpose it would be desirable if the TCR had a high affinity and/or a slow off-rate for the peptide-HLA complex.

[0003] Anikeeva et al. (Journal of Immunological methods 277 (2003) 75-86) describes a soluble T cell receptor derived from a human CD8^{+} cytotoxic T lymphocyte clone D3 that recognises the immunodominant HIV Gag peptide SLYNTVATL (SL9) in association with major histocompatiability complex class 1 protein HLA-A2.

## Brief Description of the Invention

[0004] The invention provides in a first aspect a T-cell receptor (TCR) comprising the TCR alpha chain variable region of Fig 1a and the TCR beta chain variable region of Fig 1b.

[0005] The TCR of the first aspect may comprise the alpha chain of Fig 3a and the beta chain of Fig 3b.

[0006] The TCR of the first aspect may comprise the alpha chain of Fig 5a and the beta chain of Fig 5b.

[0007] The TCR of the first aspect may lack transmembrane and cytoplasmic regions.

[0008] In a second aspect the present invention provides a cell harbouring a TCR expression vector which comprises nucleic acid encoding the TCR alpha chain of Fig 16a and the TCR beta chain of Fig 16b.

[0009] In a third aspect, the present invention provides a T cell comprising an expression vector comprising a nucleic acid encoding a TCR according to the first aspect of the present invention.

## Detailed Description of the Invention

[0010] The invention provides in a first aspect; a T-cell receptor (TCR) comprising the TCR alpha chain variable region of SEQ ID NO: 1 and the TCR beta chain variable region of SEQ ID NO:2. The TCR of the first aspect may comprise the alpha chain of SEQ ID NO:5 and the beta chain of SEQ ID NO:6 or may comprise the alpha chain of SEQ ID NO:9 and the beta chain of SEQ ID NO: 10. The TCR of the first aspect may lack transmembrane and cytoplasmic regions.

[0011] Phage display provides one means by which libraries of TCR variants can be generated. Methods suitable for the phage display and subsequent screening of libraries of TCR variants each containing a non-native disulfide interchain bond are detailed in (Li et al., (2005) Nature Biotech 23 (3): 349-354) and WO 2004/04404.

[0012] Native TCRs exist in heterodimeric αβ or γδ forms. However, recombinant TCRs consisting of a single TCR α or TCR β chain have previously been shown to bind to peptide MHC molecules.

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**[0013]** In one embodiment the TCR of the invention comprise both an α chain variable domain and an TCR P chain variable domain.

**[0014]** Unless stated to the contrary, the TCR amino acid sequences herein are generally provided including an N-terminal methionine (Met or M) residue. As will be known to those skilled in the art this residue may be removed during the production of recombinant proteins. As will also be obvious to those skilled in the art, it may be possible to truncate the sequences provided at the C-terminus and/or N-terminus thereof, by 1, 2, 3, 4, 5 or more residues, without substantially affecting the pMHC binding characteristics of the TCR, all such trivial variants are encompassed by the present invention.

**[0015]** As used herein the term "variable region" is understood to encompass all amino acids of a given TCR which are not included within the constant domain as encoded by the TRAC gene for TCR α chains and either the TRBC1 or TRBC2 genes for TCR P chains. (T cell receptor Factsbook, (2001) LeFranc and LeFranc, Academic Press, ISBN 0-12-441352-8)

**[0016]** As used herein the term "variable domain" is understood to encompass all amino acids of a given TCR which are encoded by a TRAV gene for TCR α chains and a TRBV gene for TCR P chains. (T cell receptor Factsbook, (2001) LeFranc and LeFranc, Academic Press, ISBN 0-12-441352-8)

**[0017]** As is known to those skilled in the art, part of the diversity of the TCR repertoire is due to variations which occur in the amino acid encoded by the codon at the boundary between the variable region, as defined herein, and the constant domain. For example, the codon that is present at this boundary in the parental HIV Gag TCR sequence results in the presence of the Histidine (H) residue at the C-terminal of the variable region sequences herein. This Histidine replaces the N-terminal Asparagine (N) residue encoded by the TRAC gene shown in Figure 8a.

**[0018]** Native TCRs exist in heterodimeric αβ or γδ forms. However, recombinant TCRs consisting of αα or ββ homodimers have previously been shown to bind to peptide MHC molecules.

**[0019]** TCRs of the invention may further comprise the alpha chain constant domain amino acid sequence shown in Figure 8a (SEQ ID NO: 19) and one of the beta chain amino acid constant domain sequences shown in Figures 8b and 8c (SEQ ID NOs: 20 and 21) or phenotypically silent variants thereof.

**[0020]** As used herein the term "phenotypically silent variants" is understood to refer to those TCRs which have a $K_D$ for the said SLYNTVATL-HLA-A*0201 complex of less than or equal to 1μM and/or have an off-rate ($k_{off}$) of $1 \times 10^{-3}$ $S^{-1}$ or slower. For example, as is known to those skilled in the art, it may be possible to produce TCRs that incorporate minor changes in the constant domain and/or variable regions thereof compared to those detailed above without altering the affinity and/or off-rate for the interaction with the SLYNTVATL-HLA-A*0201 complex. Such trivial variants are included in the scope of this invention. Those TCRs in which one or more conservative substitutions have been made also form part of this invention.

**[0021]** In one broad aspect, the TCRs of the invention are in the form of either single chain TCRs (scTCRs) or dimeric TCRs (dTCRs) as described in WO 04/033685 and WO 03/020763.

**[0022]** A suitable scTCR form comprises a first segment constituted by an amino acid sequence corresponding to a TCR α chain variable region, a second segment constituted by an amino acid sequence corresponding to a TCR β chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR β chain constant domain extracellular sequence, and a linker sequence linking the C terminus of the first segment to the N terminus of the second segment.

**[0023]** Alternatively the first segment may be constituted by an amino acid sequence corresponding to a TCR β chain variable region, the second segment may be constituted by an amino acid sequence corresponding to a TCR α chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant domain extracellular sequence

**[0024]** The above scTCRs may further comprise a disulfide bond between the first and second chains, said disulfide bond being one which has no equivalent in native αβT cell receptors, and wherein the length of the linker sequence and the position of the disulfide bond being such that the variable domain sequences of the first and second segments are mutually orientated substantially as in native αβ T cell receptors.

**[0025]** More specifically the first segment may be constituted by an amino acid sequence corresponding to a TCR α chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant domain

extracellular sequence, the second segment may be constituted by an amino acid sequence corresponding to a TCR β chain variable region fused to the N terminus of an amino acid sequence corresponding to TCR β chain constant domain extracellular sequence, and a disulfide bond may be provided between the first and second chains, said disulfide bond being one which has no equivalent in native αβ T cell receptors.

[0026] In the above scTCR forms, the linker sequence may link the C terminus of the first segment to the N terminus of the second segment, and may have the formula -PGGG-(SGGGG)$_n$-P- wherein n is 5 or 6 and P is proline, G is glycine and S is serine.

-PGGG-SGGGGSGGGGSGGGGSGGGGSGGGG-P (SEQ ID NO: 17)

-PGGG-SGGGGSGGGGSGGGGSGGGGSGGGGSGGGG-P (SEQ ID NO: 18)

[0027] A suitable dTCR form of the TCRs of the present invention comprises a first polypeptide wherein a sequence corresponding to a TCR α chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant domain extracellular sequence, and a second polypeptide wherein a sequence corresponding to a TCR β chain variable region sequence fused to the N terminus a sequence corresponding to a TCR β chain constant domain extracellular sequence, the first and second polypeptides being linked by a disulfide bond which has no equivalent in native αβ T cell receptors.

[0028] The first polypeptide may comprise a TCR α chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant domain extracellular sequence, and a second polypeptide wherein a sequence corresponding to a TCR β chain variable region sequence is fused to the N terminus a sequence corresponding to a TCR β chain constant domain extracellular sequence, the first and second polypeptides being linked by a disulfide bond between cysteine residues substituted for Thr 48 of exon 1 of TRAC*01 and Ser 57 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof. ("TRAC" etc. nomenclature herein as per T cell receptor Factsbook, (2001) LeFranc and LeFranc, Academic Press, ISBN 0-12-441352-8)

[0029] The dTCR or scTCR form of the TCRs of the invention may have amino acid sequences corresponding to human αβ TCR extracellular constant domain and variable region sequences, and a disulfide bond may link amino acid residues of the said constant domain sequences, which disulfide bond has no equivalent in native TCRs. The disulfide bond is between cysteine residues corresponding to amino acid residues whose β carbon atoms are less than 0.6 nm apart in native TCRs, for example between cysteine residues substituted for Thr 48 of exon 1 of TRAC*01 and Ser 57 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof. Other sites where cysteines can be introduced to form the disulfide bond are the following residues in exon 1 of TRAC*01 for the TCR α chain and TRBC1*01 or TRBC2*01 for the TCR β chain:

| TCR α chain | TCR β chain | Native β carbon separation (nm) |
|---|---|---|
| Thr 45 | Ser 77 | 0.533 |
| Tyr 10 | Ser 17 | 0.359 |
| Thr 45 | Asp 59 | 0.560 |
| Ser 15 | Glu 15 | 0.59 |

[0030] In addition to the non-native disulfide bond referred to above, the dTCR or scTCR form of the TCRs of the invention may include a disulfide bond between residues corresponding to those linked by a disulfide bond in native TCRs.

[0031] The dTCR or scTCR form of the TCRs of the invention preferably does not contain a sequence corresponding to transmembrane or cytoplasmic sequences of native TCRs.

[0032] TCRs of the invention bind strongly to the SLYNTVATL-HLA-A2*0201. These TCRs also bind to an altered, but still useful, extent to naturally occurring variants of the HIV Gag-derived SLYNTVATL when loaded by HLA-A*0201. Variants of the SLYNTVATL which have been isolated from AIDs patients include the following ( Sewell et al., (1997) Eur J Immunol. 27: 2323-2329):

SLFNTVATL

SLFNTVAVL

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SL<u>S</u>NTVATL

SS<u>F</u>NTVATL

SL<u>L</u>NTVATL

SLYNT<u>I</u>ATL

SLYNT<u>IAV</u>L

SL<u>F</u>NT<u>I</u>ATL

SL<u>F</u>NT<u>IAV</u>L

SL<u>F</u>N<u>F</u>VATL

**[0033]** The mutated amino acids are underlined.

*PEGylated TCR Monomers*

**[0034]** The TCR of the invention may be associated with at least one polyalkylene glycol chain(s). This association may be cause in a number of ways known to those skilled in the art. The polyalkylene chain(s) may be covalently linked to the TCR. In a further embodiment the polyethylene glycol chains of the present aspect of the invention comprise at least two polyethylene repeating units.

*Multivalent TCR Complexes*

**[0035]** One aspect of the invention may provide a multivalent TCR complex comprising at least two TCRs of the invention. At least two TCR molecules may be are linked via linker moieties to form multivalent complexes. Preferably the complexes are water soluble, so the linker moiety should be selected accordingly. Furthermore, it is preferable that the linker moiety should be capable of attachment to defined positions on the TCR molecules, so that the structural diversity of the complexes formed is minimised. A TCR complex may be provided wherein the polymer chain or peptidic linker sequence extends between amino acid residues of each TCR which are not located in a variable region sequence of the TCR.

**[0036]** The complexes may be for use in medicine, the linker moieties should be chosen with due regard to their pharmaceutical suitability, for example their immunogenicity.

**[0037]** Examples of linker moieties which fulfil the above desirable criteria are known in the art, for example the art of linking antibody fragments.

**[0038]** There are two classes of linker that are preferred for use in the production of multivalent TCR molecules of the present invention. A TCR complex of the invention in which the TCRs are linked by a polyalkylene glycol chain provides one embodiment of the present aspect.

**[0039]** The first are hydrophilic polymers such as polyalkylene glycols. The most commonly used of this class are based on polyethylene glycol or PEG, the structure of which is shown below.

$$HOCH_2CH_2O\ (CH_2CH_2O)_n\text{-}CH_2CH_2OH$$
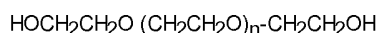
**[0040]** Wherein n is greater than two. However, others are based on other suitable, optionally substituted, polyalkylene glycols include polypropylene glycol, and copolymers of ethylene glycol and propylene glycol.

**[0041]** Such polymers may be used to treat or conjugate therapeutic agents, particularly polypeptide or protein therapeutics, to

achieve beneficial changes to the PK profile of the therapeutic, for example reduced renal clearance, improved plasma half-life, reduced immunogenicity, and improved solubility. Such improvements in the PK profile of the PEG-therapeutic conjugate are believe to result from the PEG molecule or molecules forming a 'shell' around the therapeutic which sterically hinders the reaction with the immune system and reduces proteolytic degradation. (Casey et al, (2000) Tumor Targetting 4 235-244 ) The size of the hydrophilic polymer used my in particular be selected on the basis of the intended therapeutic use of the TCR complex. There are numerous review papers and books that detail the use of PEG and similar molecules in pharmaceutical formulations. For example, see Harris (1992) Polyethylene Glycol Chemistry - Biotechnical and Biomedical Applications, Plenum, New York, NY. or Harris & Zalipsky (1997) Chemistry and Biological Applications of Polyethylene Glycol ACS Books, Washington, D.C.

[0042]  The polymer used can have a linear or branched conformation. Branched PEG molecules, or derivatives thereof, can be induced by the addition of branching moieties including glycerol and glycerol oligomers, pentaerythritol, sorbitol and lysine.

[0043]  Usually, the polymer will have a chemically reactive group or groups in its structure, for example at one or both termini, and/or on branches from the backbone, to enable the polymer to link to target sites in the TCR. This chemically reactive group or groups may be attached directly to the hydrophilic polymer, or there may be a spacer group/moiety between the hydrophilic polymer and the reactive chemistry as shown below:

Reactive chemistry-Hydrophilic polymer-Reactive chemistry

Reactive chemistry-Spacer-Hydrophilic polymer-Spacer-Reactive chemistry

[0044]  The spacer used in the formation of constructs of the type outlined above may be any organic moiety that is a non-reactive, chemically stable, chain, Such spacers include, by are not limited to the following:

$-(CH_2)_n-$

wherein n = 2 to 5

$-(CH_2)_3NHCO(CH_2)_2$

[0045]  A TCR complex in which a divalent alkylene spacer radical is located between the polyalkylene glycol chain and its point of attachment to
a TCR of the complex provides a further embodiment of the present aspect.

[0046]  A TCR complex in which the polyalkylene glycol chain comprises at least two polyethylene glycol repeating units is described.

[0047]  There are a number of commercial suppliers of hydrophilic polymers linked, directly or via a spacer, to reactive chemistries that may be of use in the present invention. These suppliers include Nektar Therapeutics (CA, USA), NOF Corporation (Japan), Sunbio (South Korea) and Enzon Pharmaceuticals (NJ, USA).

[0048]  Commercially available hydrophilic polymers linked, directly or via a spacer, to reactive chemistries that may be of use in the present invention include, but are not limited to, the following:

| PEG linker Description | Source of PEG | Catalogue Number |
|---|---|---|
| TCR Monomer attachment | | |
| 5K linear (Maleimide) | Nektar | 2D2MOHO1 |
| 20K linear (Maleimide) | Nektar | 2D2MOPO1 |
| 20K linear (Maleimide) | NOF Corporation | SUNBRIGHT ME-200MA |
| 20K branched (Maleimide) | NOF Corporation | SUNBRIGHT GL2-200MA |
| 30K linear (Maleimide) | NOF Corporation | SUNBRIGHT ME-300MA |
| 40K branched PEG (Maleimide) | Nektar | 2D3XOTO1 |

| PEG linker Description | Source of PEG | Catalogue Number |
|---|---|---|
| 5K-NP linear (for Lys attachment) | NOF Corporation | SUNBRIGHT MENP-50H |
| 10K-NP linear (for Lys attachment) | NOF Corporation | SUNBRIGHT MENP-10T |
| 20K-NP linear (for Lys attachment) | NOF Corporation | SUNBRIGHT MENP-20T |
| **TCR dimer linkers** | | |
| 3.4K linear (Maleimide) | Nektar | 2D2DOFO2 |
| 5K forked (Maleimide) | Nektar | 2D2DOHOF |
| 10K linear (with orthopyridyl ds-linkers in place of Maleimide) | Sunbio | |
| 20K forked (Maleimide) | Nektar | 2D2DOPOF |
| 20K linear (Maleimide) | NOF Corporation | |
| 40K forked (Maleimide) | Nektar | 2D3XOTOF |
| **Higher order TCR multimers** | | |
| 15K, 3 arms, $Mal_3$ (for trimer) | Nektar | OJOONO3 |
| 20K, 4 arms, $Mal_4$ (fortetramer) | Nektar | OJOOPO4 |
| 40 K, 8 arms, $Mal_8$ (for octamer) | Nektar | OJOOTO8 |

[0049] A wide variety of coupling chemistries can be used to couple polymer molecules to protein and peptide therapeutics. The choice of the most appropriate coupling chemistry is largely dependant on the desired coupling site. For example, the following coupling chemistries have been used attached to one or more of the termini of PEG molecules (Source: Nektar Molecular Engineering Catalogue 2003):

N-maleimide

Vinyl sulfone

Benzotriazole carbonate

Succinimidyl proprionate

Succinimidyl butanoate

Thio-ester

Acetaldehydes

Acrylates

Biotin

Primary amines

[0050] As stated above non-PEG based polymers also provide suitable linkers for multimerising the TCRs of the present invention. For example, moieties containing maleimide termini linked by aliphatic chains such as BMH and BMOE (Pierce, products Nos. 22330 and 22323) can be used.

[0051] Peptidic linkers are the other class of TCR linkers. These linkers are comprised of chains of amino acids, and function to produce simple linkers or multimerisation domains onto which TCR molecules can be attached. The biotin / streptavidin system has previously been used to produce TCR tetramers (see WO/99/60119) for in-vitro binding studies. However, strepavidin is a microbially-derived polypeptide and as such not ideally suited to use in a therapeutic.

[0052] A TCR complex in which the TCRs are linked by a peptidic linker derived from a human multimerisation domain is described.

**[0053]** There are a number of human proteins that contain a multimerisation domain that could be used in the production of multivalent TCR complexes. For example the tetramerisation domain of p53 which has been utilised to produce tetramers of scFv antibody fragments which exhibited increased serum persistence and significantly reduced off-rate compared to the monomeric scFV fragment. (Willuda et al. (2001) J. Biol. Chem. 276 (17) 14385-14392) Haemoglobin also has a tetramerisation domain that could potentially be used for this kind of application.

**[0054]** A multivalent TCR complex comprising at least two TCRs is described, wherein at least one of said TCRs is associated with a therapeutic agent.

**[0055]** A TCR (or multivalent complex thereof) of the present invention may alternatively or additionally comprise a reactive cysteine at the C-terminal or N-terminal of the alpha or beta chains thereof.

*Diagnostic and therapeutic Use*

**[0056]** The TCR of the invention may be associated with a therapeutic agent or detectable moiety. For example, said therapeutic agent or detectable moiety may be covalently linked to the TCR.

**[0057]** Said therapeutic agent or detectable moiety may be covalently linked to the C-terminus of one or both TCR chains.

**[0058]** The scTCR or one or both of the dTCR chains of TCRs of the present invention may be labelled with an detectable moiety, for example a label that is suitable for diagnostic purposes. Such labelled TCRs are useful in a method for detecting a SLYNTVATL-HLA-A*0201 complex which method comprises contacting the TCR ligand with a TCR (or a multimeric high affinity TCR complex) which is specific for the TCR ligand; and detecting binding to the TCR ligand. In tetrameric TCR complexes formed for example, using biotinylated heterodimers, fluorescent streptavidin can be used to provide a detectable label. Such a fluorescently-labelled TCR tetramer is suitable for use in FACS analysis, for example to detect antigen presenting cells carrying the SLYNTVATL-HLA-A*0201 complex for which these high affinity TCRs are specific.

**[0059]** Another manner in which the soluble TCRs of the present invention may be detected is by the use of TCR-specific antibodies, in particular monoclonal antibodies. There are many commercially available anti-TCR antibodies, such as αF1 and βF1, which recognise the constant domains of the α and β chains, respectively.

**[0060]** A TCR (or multivalent complex thereof) of the present invention may alternatively or additionally be associated with (e.g. covalently or otherwise linked to) a therapeutic agent which may be, for example, a toxic moiety for use in cell killing, or an immune effector molecule such as an interleukin or a cytokine. A multivalent TCR complex of the invention may have enhanced binding capability for a TCR ligand compared to a non-multimeric wild-type or T cell receptor heterodimer of the invention. Thus, the multivalent TCR complexes according to the invention are particularly useful for tracking or targeting cells presenting SLYNTVATL-HLA-A*0201 complexes *in vitro* or *in vivo,* and are also useful as intermediates for the production of further multivalent TCR complexes having such uses. These TCRs or multivalent TCR complexes may therefore be provided in a pharmaceutically acceptable formulation for use *in vivo.*

**[0061]** A method for delivering a therapeutic agent to a target cell is described, which method comprises contacting potential target cells with a TCR or multivalent TCR complex under conditions to allow attachment of the TCR or multivalent TCR complex to the target cell, said TCR or multivalent TCR complex being specific for the SLYNTVATL-HLA-A*0201 complex and having the therapeutic agent associated therewith.

**[0062]** In particular, the soluble TCR or multivalent TCR complex can be used to deliver therapeutic agents to the location of cells presenting a particular antigen. This would be useful in many situations and, in particular, against HIV infected cells. A therapeutic agent could be delivered such that it would exercise its effect locally but not only on the cell it binds to. Thus, one particular strategy envisages cytotoxic or immuno-stimulatory molecules linked to TCRs or multivalent TCR complexes according to the invention specific for the SLYNTVATL-HLA-A*0201 complex.

**[0063]** Many therapeutic agents could be employed for this use, for instance radioactive compounds, enzymes (perforin for example) or chemotherapeutic agents (cis-platin for example). To ensure that toxic effects are exercised in the desired location the toxin could be inside a liposome linked to streptavidin so that the compound is released slowly. This will prevent damaging effects during the transport in the body and ensure that the toxin has maximum effect after binding of the TCR to the relevant antigen presenting cells.

**[0064]** Other suitable therapeutic agents include:

- small molecule cytotoxic agents, i.e. compounds with the ability to kill mammalian cells having a molecular weight of less than 700 daltons. Such compounds could also contain toxic metals capable of having a cytotoxic effect. Furthermore, it is to be understood that these small molecule cytotoxic agents also include pro-drugs, i.e. compounds that decay or are converted under physiological conditions to release cytotoxic agents. Examples of such agents include cis-platin, maytansine derivatives, rachelmycin, calicheamicin, docetaxel, etoposide, gemcitabine, ifosfamide, irinotecan, melphalan, mitoxantrone, sorfimer sodiumphotofrin II, temozolmide, topotecan, trimetreate glucuronate, auristatin E vincristine and doxorubicin;
- peptide cytotoxins, i.e. proteins or fragments thereof with the ability to kill mammalian cells. Including but not limited to, ricin, diphtheria toxin, pseudomonas bacterial exotoxin A, DNAase and RNAase;
- radio-nuclides, i.e. unstable isotopes of elements which decay with the concurrent emission of one or more of $\alpha$ or $\beta$ particles, or $\gamma$ rays. including but not limited to, iodine 131, rhenium 186, indium 111, yttrium 90, bismuth 210 and 213, actinium 225 and astatine 213; chelating agents may be used to facilitate the association of these radio-nuclides to the high affinity TCRs, or multimers thereof;

- prodrugs, including but not limited to, antibody directed enzyme pro-drugs;
- immuno-stimulants, i.e. moieties which stimulate immune response. Including but not limited to, cytokines such as IL-2 and IFN, Superantigens and mutants thereof, TCR-HLA fusions and chemokines such as IL-8, platelet factor 4, melanoma growth stimulatory protein, etc, antibodies or fragments thereof, complement activators, xenogeneic protein domains, allogeneic protein domains, viral/bacterial protein domains, viral/bacterial peptides and anti-T cell determinant antibodies (e.g. anti-CD3 or anti-CD28) or antibody analogues such as Nanobodies™ and Affybodies™.

**[0065]** Soluble TCRs or multivalent TCR complexes may be linked to an enzyme capable of converting a prodrug to a drug. This allows the prodrug to be converted to the drug only at the site where it is required (i.e. targeted by the sTCR).

**[0066]** It is expected that the high affinity SLYNTVATL (SEQ ID NO: 16)-HLA-A*0201 specific TCRs disclosed herein may be used in methods for the diagnosis and treatment of AIDS.

**[0067]** For treatment, therapeutic agent localisation in the vicinity of HIV infected (CD4$^+$) cells would enhance the effect of toxins or immunostimulants. For vaccine delivery, the vaccine antigen could be localised in vicinity of antigen presenting cells, thus enhancing the efficacy of the antigen. The method can also be applied for imaging purposes.

**[0068]** Described herein is a membrane preparation comprising a TCR of the invention. Said membrane preparation may be prepared from cells or may comprise a synthetic membrane.

**[0069]** Described herein is a cell harbouring an expression vector comprising nucleic acid encoding a TCR of the invention. For example, said cell may be a T cell.

**[0070]** Also described herein is a pharmaceutical composition comprising:

a TCR or a multivalent TCR complex (optionally associated with a therapeutic agent), or a membrane preparation comprising a TCR, or a plurality of cells harbouring an expression vector comprising nucleic acid encoding a TCR of the invention, together with a pharmaceutically acceptable carrier;

**[0071]** Described herein is a method of treatment of AIDS comprising administering to a subject suffering such AIDS an effective amount of a TCR or a multivalent TCR complex, or a membrane preparation comprising a TCR, or a plurality of cells harbouring an expression vector comprising nucleic acid encoding a TCR of the invention. The use of a TCR or a multivalent TCR complex, or a membrane preparation comprising a TCR of the invention, or a plurality of cells harbouring an expression vector comprising nucleic acid encoding a TCR of the invention, in the preparation of a composition for the treatment of AIDS is described. Further uses and methods are described wherein the TCR, or multivalent TCR complex, or a membrane preparation comprising a TCR is administered in a form which is associated with a therapeutic agent. The cells harbouring an expression vector comprising nucleic acid encoding a TCR of the invention may be CD8$^+$ T cells.

[0072] Therapeutic or imaging TCRs will usually be supplied as part of a sterile, pharmaceutical composition which will normally include a pharmaceutically acceptable carrier. This pharmaceutical composition may be in any suitable form, (depending upon the desired method of administering it to a patient). It may be provided in unit dosage form, will generally be provided in a sealed container and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use. It may include a plurality of said unit dosage forms.

[0073] Without wishing to be limited by theory, it is expected that the TCRs of the invention will provide effective targeting agents capable of delivering therapeutic agents such as immunostimulants and/or cytotoxic agents to HIV infected (CD4$^+$) cells. In particular, it is expected that the administration of the TCRs of the present invention when associated with immunostimulants and/or cytotoxic agents in combination with conventional antiretrovirus drug therapies and/or IL-2 treatment will be able to target HIV infected cells.

[0074] The following is a list of anti-retroviral drugs currently approved for use in the US:

Agenerase (amprenavir) - protease inhibitor

Combivir -combination of Retrovir (300mg) and Epivir (150mg)

Crixivan (indinavir) - protease inhibitor

Epivir (3tc / lamivudine) - nucleoside analog reverse transcriptase inhibitor

Epzicom ( a combination of 2 nucleoside reverse transcriptase inhibitors (NRTIs in the same pill; 600mg of Ziagen (abacavir) and 300mg of Epivir (3TC).

Emtriva [ emtricitabine (FTC)]

Fortovase (saquinavir) - protease inhibitor

Fuzeon (enfuvirtide) - Fusion inhibitor

Hivid (ddc / zalcitabine) - nucleoside analog reverse transcriptase inhibitor

Invirase (saquinavir) - protease inhibitor

Kaletra (lopinavir) - protease inhibitor

Lexiva (Fosamprenavir) - Protease Inhibitor approved 10/20/03

Norvir (ritonavir) - protease inhibitor

Rescriptor (delavirdine) - non nucleoside analog reverse transcriptase inhibitor

Retrovir, AZT (zidovudine) - nucleoside analog reverse transcriptase inhibitor

Reyataz (atazanavir; BMS-232632) - protease inhibitor

Sustiva (efavirenz) - non nucleoside analog reverse transcriptase inhibitor

Trizivir (3 non nucleosides in one tablet; abacavir + zidovudine + lamivudine

Truvada (Emtricitabine + Tenofovir DF )

Videx (ddI / didanosine) nucleoside analog reverse transcriptase inhibitor

Videx EC; (ddI / didanosine) nucleoside analog reverse transcriptase inhibitor;

Viracept (nelfinavir) - protease inhibitor

Viramune (nevirapine) - non nucleoside analog Reverse transcriptase inhibitor

Viread (tenofovir disoproxil fumarate) Nucleotide Reverse transcriptase inhibitor

(Adenosine Class)

Zerit (d4t / stavudine) - nucleoside analog reverse transcriptase inhibitor

Ziagen (abacavir) - nucleoside analog reverse transcriptase inhibitor

**[0075]** The pharmaceutical composition may be adapted for administration by any appropriate route, for example parenteral, transdermal or via inhalation, preferably a parenteral (including subcutaneous, intramuscular, or, most preferably intravenous) route. Such compositions may be prepared by any method known in the art of pharmacy, for example by mixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

**[0076]** Dosages of the substances can vary between wide limits, depending upon the disease or disorder to be treated, the age and condition of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used.

*Additional Aspects*

**[0077]** A scTCR or dTCR (which preferably is constituted by constant and variable sequences corresponding to human sequences) of the present invention may be provided in substantially pure form, or as a purified or isolated preparation. For example, it may be provided in a form which is substantially free of other proteins.

**[0078]** The sequence(s) of the nucleic acid or nucleic acids encoding the TCRs of the invention may be altered so as to optimise the level of expression obtained in the host cell. The host cell may be any appropriate prokaryotic or eukaryotic cell. For example, the host cell may be an *E.coli* cell or a human T cell. The alterations made to these genetic sequences are silent, that is they do not alter the amino acid sequence encoded. There are a number of companies which offer such expression optimisation services, including, GeneArt, Germany.

**[0079]** Described herein is a method of producing a high affinity TCR having the property of binding to SLYNTVATL-HLA-A*0201.CHARACTERISED IN THAT the TCR (i) comprises at least one TCR α chain variable domain and/or at least one TCR β chain variable domain and (ii) has a $K_D$ for the said SLYNTVATL-HLA-A*0201 complex of less than or equal to 1μM and/or an off-rate ($k_{off}$) for the SLYNTVATL-HLA-A*0201 complex of $1 \times 10^{-3}$ $S^{-1}$ or slower, wherein the method comprises:

1. (a) the production of a TCR comprising the α and β chain variable domains of the parental HIV Gag TCR wherein one or both of the α and β chain variable domains comprise a mutation(s) in one or more of the amino acids identified in claims 7 and 8;
2. (b) contacting said mutated TCR with SLYNTVATL-HLA-A*0201 under conditions suitable to allow the binding of the TCR to SLYNTVATL-HLA-A*0201;

and measuring the $K_D$ and/or $k_{off}$ of the interaction.

**[0080]** Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis.* The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

**Examples**

**[0081]** The invention is further described in the following examples, which do not limit the scope of the invention in any way.

**[0082]** Reference is made in the following to the accompanying drawings in which:

Figure 1a and 1b details the alpha chain variable domain amino acid and beta chain variable domain amino acid sequences of the parental HIV Gag TCR respectively.

Figures 2a and 2b show respectively the DNA sequence of soluble versions of the parental HIV Gag TCR α and β chains.

Figures 3a and 3b show respectively the HIV Gag TCR α and β chain extracellular amino acid sequences produced from the DNA sequences of Figures 2a and 2b.

Figures 4a and 4b show respectively the DNA sequence of soluble versions of the HIV Gag TCR α and β chains mutated to enocode additional cysteine residues to form a non-native disulfide bond. The mutated codon is indicated by shading and The introduced restriction enzyme recognition sites are underlined..

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Figures 5a and 5b show respectively the HIV Gag TCR α and β chain extracellular amino acid sequences produced from the DNA sequences of Figures 4a and 4b. The introduced cysteine in each chain is indicated by shading.

Figure 6 details the alpha chain variable domain amino acid sequences of the high affinity HIV Gag TCR variants.

Figure 7 details the beta chain variable domain amino acid sequences of the high affinity HIV Gag TCR variants.

Figure 8a details the amino acid sequence of a soluble portion of TRAC.

Figure 8b details the amino acid sequence of a soluble portion of TRBC1.

Figure 8c details the amino acid sequence of a soluble portion of TRBC2.

Figure 9 details the DNA sequence of the pEX954 plasmid.

Figure 10 details the DNA sequence of the pEX821 plasmid.

Figure 11 details the beta chain amino acid sequences of the parental soluble HIV Gag TCR variant fused via a peptide linker to wild-type human IL-2. The amino acids of the linker and IL-2 are indicated in italics.

Figure 12 provides the Biacore response curves generated for the interaction of the soluble disulfide-linked parental HIV Gag TCR and the SLYNTVATL-HLA-A*0201 complex.

Figure 13 provides a plasmid map of the pEX954 plasmid.

Figure 14 provides a plasmid map of the pEX821 plasmid.

Figure 15a provides the full-length DNA sequence of the parental HIV Gag TCR α chain optimised for expression in human T cells.

Figure 15b provides the full-length DNA sequence of the parental HIV Gag TCR β chain optimised for expression in human T cells.

Figure 16a provides the full-length amino acid sequence of the parental HIV Gag TCR α chain.

Figure 16b provides the full-length amino acid sequence of the parental HIV Gag TCR β chain optimised for expression in human T cells.

Figures 17a provides FACS analysis data for untransduced control CD8^{+} T cells.

Figure 17b provides FACS analysis data demonstrating expression of the parental HIV Gag TCR on the surface of transduced CD8^{+} T cells.

Figures 18a and 18b provide the amino acids sequences of the alpha and beta chains of a soluble disulfide-linked high affinity c11c6 HIV Gag TCR.respectively.

Figure 19 demonstrates the ability of soluble disulfide-linked high affinity c 11c6 HIV Gag TCRs to inhibit the activation of the SLYNTVATL-HLA-A*0201 reactive OX84 polyclonal T cell line in the presence of To cells infected with HIV as measured by IFN-γ and TNF-α production.

Figure 20 demonstrates the ability of soluble disulfide-linked high affinity c11c6 HIV Gag TCRs to inhibit the activation of the SLYNTVATL-HLA-A*0201 reactive OX84 polyclonal T cell line in the presence of SLYNTVATL peptide-pulsed uninfected To cells as measured by IFN-γ and TNF-α production.

Figure 21 demonstrates the ability of soluble disulfide-linked high affinity c11c6 HIV Gag TCRs to stain SLYNTVATL peptide-pulsed T2 cells.

***Example 1 - production of soluble disulfide-linked TCRs comprising the parental HIV Gag TCR variable regions***

[0083] Figures 4a and 4b provide the DNA sequences of soluble disulfide-linked alpha beta chains from a parental TCR which is

specific for the SLYNTVATL-HLA-A*0201 complex. These DNA sequences can be synthesis de-novo by a number of contract research companies, for example GeneArt (Germany). Restriction enzyme recognition sites are also added to these DNA sequences in order to facilitate ligation of these DNA sequences into the pGMT7-based expression plasmids, which contain the T7 promoter for high level expression in E.coli strain BL21-DE3(pLysS) (Pan et al., Biotechniqates (2000) 29 (6): 1234-8)

**[0084]** The TCR alpha chain sequences contain introduced *ClaI* and *SalII* restriction enzyme recognition sites and this sequence was ligated into pEX954 (see Figures 9 and 13 ) cut with *ClaI* and *XhoI.*

**[0085]** The TCR beta chain sequences contain introduced *AseI* and *AgeI* restriction enzyme recognition sites and were ligated into pEX821 (see Figures 10 and 14) cut with *NdeI/AgeI.*

*Restriction enzyme recognition sites as introduced into DNA encoding the TCR chains*

**[0086]**

*ClaI-* ATCGAT

*SalII-* GTCGAC

*AseI-* ATTAAT

*AgeI-* ACCGGT

*Ligation*

**[0087]** The cut TCR alpha and beta chain DNA and cut vector were ligated using a rapid DNA ligation kit (Roche) following the manufacturers instructions.

**[0088]** Ligated plasmids were transformed into competent E.coli strain XL1-blue cells and plated out on LB/agar plates containing 100mg/ml ampicillin. Following incubation overnight at 37°C, single colonies were picked and grown in 10 ml LB containing 100mg/ml ampicillin overnight at 37°C with shaking. Cloned plasmids were purified using a Miniprep kit (Qiagen) and the insert was sequenced using an automated DNA sequencer (Lark Technologies).

**[0089]** Figures 5a and 5b show respectively the soluble disulfide linked parental HIV gag TCR α and β chain extracellular amino acid sequences produced from the DNA sequences of Figures 4a and 4b

*Example 2- Production of high affinity variants of the soluble disulfide linked HIV Gag TCR*

**[0090]** The soluble disulfide-linked native HIV Gag TCR produced as described in Example 1 can be used a template from which to produce the TCRs of the invention which have an increased affinity for the SLYNTVATL (SEQ ID NO: 16) -HLA-A*0201 complex.

**[0091]** Phage display is one means by which libraries of HIV Gag TCR variants can be generated in order to identify high affinity mutants. For example, the TCR phage display and screening methods described in (Li et al., (2005) Nature Biotech 23 (3): 349-354) can be adapted and applied to HIV Gag TCRs.

**[0092]** The amino sequences of the mutated TCR alpha and beta chain variable domains which, when combined with an appropriate TCR chain, demonstrate high affinity for the SLYNTVATL-HLA-A*0201 complex, are listed in Figures 6 and 7 respectively. (SEQ ID Nos: 11-13 and 14-15 respectively) As is known to those skilled in the art the necessary codon changes required to produce these mutated chains can be introduced into the DNA encoding these chains by site-directed mutagenesis. (QuickChange™ Site-Directed Mutagenesis Kit from Stratagene)

**[0093]** Briefly, this is achieved by using primers that incorporate the desired codon change(s) and the plasmids containing the

relevant TCR chain DNA as a template for the mutagenesis:

**[0094]** Mutagenesis was carried out using the following conditions : 50ng plasmid template, 1µl of 10mM dNTP, 5µl of 10x Pfu DNA polymerase buffer as supplied by the manufacturer, 25 pmol of fwd primer, 25 pmol of rev primer, 1µl pfu DNA polymerase in total volume 50 µl. After an initial denaturation step of 2 mins at 95C, the reaction was subjected to 25 cycles of denaturation (95C, 10 secs), annealing (55C 10 secs), and elongation (72C, 8 mins). The resulting product was digested with DpnI restriction enzyme to remove the template plasmid and transformed into E. coli strain XL1-blue. Mutagenesis was verified by sequencing.

### *Example 3 - Expression, refolding and purification of soluble TCR*

**[0095]** The expression plasmids containing the mutated α-chain and β-chain respectively as prepared in Examples 1 or 2 were transformed separately into *E.coli* strain BL21pLysS, and single ampicillin-resistant colonies were grown at 37°C in TYP (ampicillin 100µg/ml) medium to $OD_{600}$ of 0.4 before inducing protein expression with 0.5mM IPTG. Cells were harvested three hours post-induction by centrifugation for 30 minutes at 4000rpm in a Beckman J-6B. Cell pellets were re-suspended in a buffer containing 50mM Tris-HCl, 25% (w/v) sucrose, 1mM NaEDTA, 0.1% (w/v) NaAzide, 10mM DTT, pH 8.0. After an overnight freeze-thaw step, re-suspended cells were sonicated in 1 minute bursts for a total of around 10 minutes in a Milsonix XL2020 sonicator using a standard 12mm diameter probe. Inclusion body pellets were recovered by centrifugation for 30 minutes at 13000rpm in a Beckman J2-21 centrifuge. Three detergent washes were then carried out to remove cell debris and membrane components. Each time the inclusion body pellet was homogenised in a Triton buffer (50mM Tris-HCl, 0.5% Triton-X100, 200mM NaCl, 10mM NaEDTA, 0.1% (w/v) NaAzide, 2mM DTT, pH 8.0) before being pelleted by centrifugation for 15 minutes at 13000rpm in a Beckman J2-21. Detergent and salt was then removed by a similar wash in the following buffer: 50mM Tris-HCl, 1mM NaEDTA, 0.1% (w/v) NaAzide, 2mM DTT, pH 8.0. Finally, the inclusion bodies were divided into 30 mg aliquots and frozen at -70°C. Inclusion body protein yield was quantitated by solubilising with 6M guanidine-HCl and measurement with a Bradford dye-binding assay (PerBio).

**[0096]** Approximately 30mg of TCR β chain and 60mg of TCR α chain solubilised inclusion bodies were thawed from frozen stocks, samples were then mixed and the mixture diluted into 15ml of a guanidine solution (6 M Guanidine-hydrochloride, 10mM Sodium Acetate, 10mM EDTA), to ensure complete chain de-naturation. The guanidine solution containing fully reduced and denatured TCR chains was then injected into 1 litre of the following refolding buffer: 100mM Tris pH 8.5, 400mM L-Arginine, 2mM EDTA, 5mM reduced Glutathione, 0.5mM oxidised Glutathione, 5M urea, 0.2mM PMSF. The redox couple (2-mercaptoethylamine and cystamine (to final concentrations of 6.6mM and 3.7mM, respectively) were added approximately 5 minutes before addition of the denatured TCR chains. The solution was left for 5 hrs ± 15minutes. The refolded TCR was dialysed in Spectrapor 1 membrane (Spectrum; Product No. 132670) against 10 L 10 mM Tris pH 8.1 at 5°C ± 3°C for 18-20 hours. After this time, the dialysis buffer was changed to fresh 10 mM Tris pH 8.1 (10 L) and dialysis was continued at 5°C ± 3°C for another 20-22 hours.

**[0097]** sTCR was separated from degradation products and impurities by loading the dialysed refold onto a POROS 50HQ anion exchange column and eluting bound protein with a gradient of 0-500mM NaCl over 50 column volumes using an Akta purifier (Pharmacia). Peak fractions were stored at 4°C and analysed by Coomassie-stained SDS-PAGE before being pooled and concentrated. Finally, the sTCR was purified and characterised using a Superdex 200HR gel filtration column pre-equilibrated in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.5 mM EDTA, 0.05% nonidet p40). The peak eluting at a relative molecular weight of approximately 50 kDa was pooled and concentrated prior to characterisation by BIAcore surface plasmon resonance analysis.

### *Example 4 - Biacore surface plasmon resonance characterisation of sTCR binding to specific pMHC*

**[0098]** A surface plasmon resonance biosensor (Biacore 3000™ ) was used to analyse the binding of a sTCR to its peptide-MHC ligand. This was facilitated by producing single pMHC complexes (described below) which were immobilised to a streptavidin-coated binding surface in a semi-oriented fashion, allowing efficient testing of the binding of a soluble T-cell receptor to up to four different pMHC (immobilised on separate flow cells) simultaneously. Manual injection of HLA complex allows the precise level of immobilised class I molecules to be manipulated easily.

**[0099]** Biotinylated class I HLA-A*0201 molecules were refolded *in vitro* from bacterially-expressed inclusion bodies containing the constituent subunit proteins and synthetic peptide, followed by purification and *in vitro* enzymatic biotinylation (O'Callaghan et al. (1999) Anal. Biochem. 266: 9-15). HLA-A*0201-heavy chain was expressed with a C-terminal biotinylation tag which replaces the transmembrane and cytoplasmic domains of the protein in an appropriate construct. Inclusion body expression levels of ~75

mg/litre bacterial culture were obtained. The MHC light-chain or β2-microglobulin was also expressed as inclusion bodies in *E.coli* from an appropriate construct, at a level of ~500 mg/litre bacterial culture.

**[0100]** *E. coli* cells were lysed and inclusion bodies are purified to approximately 80% purity. Protein from inclusion bodies was denatured in 6 M guanidine-HCl, 50 mM Tris pH 8.1, 100 mM NaCl, 10 mM DTT, 10 mM EDTA, and was refolded at a concentration of 30 mg/litre heavy chain, 30 mg/litre β2m into 0.4 M L-Arginine-HCl, 100 mM Tris pH 8.1, 3.7 mM cystamine, 6.6 mM β-cysteamine, 4 mg/ml of the SLYNTVATL peptide required to be loaded by the HLA-A*0201 molecule, by addition of a single pulse of denatured protein into refold buffer at < 5°C. Refolding was allowed to reach completion at 4°C for at least 1 hour.

**[0101]** Buffer was exchanged by dialysis in 10 volumes of 10 mM Tris pH 8.1. Two changes of buffer were necessary to reduce the ionic strength of the solution sufficiently. The protein solution was then filtered through a 1.5μm cellulose acetate filter and loaded onto a POROS 50HQ anion exchange column (8 ml bed volume). Protein was eluted with a linear 0-500 mM NaCl gradient. HLA-A*0201-peptide complex eluted at approximately 250 mM NaCl, and peak fractions were collected, a cocktail of protease inhibitors (Calbiochem) was added and the fractions were chilled on ice.

**[0102]** Biotinylation tagged pMHC molecules were buffer exchanged into 10 mM Tris pH 8.1, 5 mM NaCl using a Pharmacia fast desalting column equilibrated in the same buffer. Immediately upon elution, the protein-containing fractions were chilled on ice and protease inhibitor cocktail (Calbiochem) was added. Biotinylation reagents were then added: 1 mM biotin, 5 mM ATP (buffered to pH 8), 7.5 mM MgCl2, and 5 μg/ml BirA enzyme (purified according to O'Callaghan et al. (1999) Anal. Biochem. 266: 9-15). The mixture was then allowed to incubate at room temperature overnight.

**[0103]** The biotinylated pHLA-A*0201 molecules were purified using gel filtration chromatography. A Pharmacia Superdex 75 HR 10/30 column was pre-equilibrated with filtered PBS and 1 ml of the biotinylation reaction mixture was loaded and the column was developed with PBS at 0.5 ml/min. Biotinylated pHLA-A*0201 molecules eluted as a single peak at approximately 15 ml. Fractions containing protein were pooled, chilled on ice, and protease inhibitor cocktail was added. Protein concentration was determined using a Coomassie-binding assay (PerBio) and aliquots of biotinylated pHLA-A*0201 molecules were stored frozen at -20°C. Streptavidin was immobilised by standard amine coupling methods.

**[0104]** Such immobilised complexes are capable of binding both T-cell receptors and the coreceptor CD8αα, both of which may be injected in the soluble phase. Specific binding of TCR is obtained even at low concentrations (at least 40μg/ml), implying the TCR is relatively stable. The pMHC binding properties of sTCR are observed to be qualitatively and quantitatively similar if sTCR is used either in the soluble or immobilised phase. This is an important control for partial activity of soluble species and also suggests that biotinylated pMHC complexes are biologically as active as non-biotinylated complexes.

**[0105]** The interactions between HIV Gag sTCR containing a novel inter-chain bond and its ligand/ MHC complex or an irrelevant HLA-peptide combination, the production of which is described above, were analysed on a Biacore 3000™ surface plasmon resonance (SPR) biosensor. SPR measures changes in refractive index expressed in response units (RU) near a sensor surface within a small flow cell, a principle that can be used to detect receptor ligand interactions and to analyse their affinity and kinetic parameters. The probe flow cells were prepared by immobilising the individual HLA-peptide complexes in separate flow cells via binding between the biotin cross linked onto β2m and streptavidin which have been chemically cross linked to the activated surface of the flow cells. The assay was then performed by passing sTCR over the surfaces of the different flow cells at a constant flow rate, measuring the SPR response in doing so.

*To measure Equilibrium binding constant*

**[0106]** Serial dilutions of the parental or mutated HIV Gag sTCR were prepared and injected at constant flow rate of 5 μl min-1 over two different flow cells; one coated with ~1000 RU of specific SLYNTVATL-HLA-A*0201 complex, the second coated with ~1000 RU of non-specific HLA-A2 -peptide complex. Response was normalised for each concentration using the measurement from the control cell. Normalised data response was plotted versus concentration of TCR sample and fitted to a hyperbola in order to calculate the equilibrium binding constant, $K_D$. (Price & Dwek, Principles and Problems in Physical Chemistry for Biochemists (2nd Edition) 1979, Clarendon Press, Oxford).

*To measure Kinetic Parameters*

**[0107]** For high affinity TCRs $K_D$ was determined by experimentally measuring the dissociation rate constant, kd, and the

association rate constant, ka. The equilibrium constant $K_D$ was calculated as kd/ka.

[0108] TCR was injected over two different cells one coated with ~300 RU of specific HLA-A2-nyeso peptide complex, the second coated with ~300 RU of non-specific HLA-A2 -peptide complex. Flow rate was set at 50 μl/min. Typically 250 μl of TCR at ~3 μM concentration was injected. Buffer was then flowed over until the response had returned to baseline. Kinetic parameters were calculated using Biaevaluation software. The dissociation phase was also fitted to a single exponential decay equation enabling calculation of half-life.

*Results*

[0109] The interaction between a soluble disulfide-linked native HIV Gag TCR (consisting of the α and β TCR chains detailed in SEQ ID NOs 9 and 10 respectively) and the SLYNTVATL-HLA-A*0201 complex was analysed using the above methods and demonstrated a $K_D$ of 85 nM and an off-rate ($k_{off}$) of 2.21 x $10^{-2}$ $S^{-1}$. (See Figure 12 for Biacore response curves)

[0110] The TCRs specified in the following table have a $K_D$ of less than or equal to 1 μM and/or a $k_{off}$ of 1 x $S^{-1}$ or slower.

| Alpha chain variable domain sequence, SEQ ID NO: | Beta chain variable domain sequence, SEQ ID NO: |
|---|---|
| 1 | 2 |
| 1 | 14 |
| 1 | 15 |
| 11 | 2 |
| 12 | 2 |
| 13 | 2 |
| 12 | 15 |
| 13 | 15 |
| 12 | 14 |
| 13 | 14 |

*Example 5 - Production of a soluble high affinity HIV Gag TCR -WT human IL-2 fusion protein*

[0111] The methods substantially as described in Examples 1 to 3 can be used to produce a soluble high affinity HIV Gag TCR-WT human IL-2 fusion protein. Briefly, the DNA encoding the desired linker and WT human IL-2 are added into the 3' end of the DNA sequence of the soluble disulfide-linked parental HIV Gag TCR beta chain immediately prior to the TAA ("Stop") codon. Figure 11 provides the amino acid sequence of a fusion protein comprising a disulfide-linked parental HIV Gag TCR beta chain fused to WT human IL-2 via linker sequence.(SEQ ID NO: 24) The linker and IL-2 portion of this fusion protein are indicated in italics. The DNA encoding this construct can then be ligated into pEX821. The soluble parental HIV Gag TCR- IL-2 fusion protein can then be expressed by combining this beta chain fusion protein with the soluble disulfide-linked parental HIV Gag alpha chain TCR chain detailed in Figure 5a (SEQ ID NO: 9) using the methods substantially as described in Example 3.

*Examples 6 - Recombinant expression of the parental HIV Gag TCR on the surface of T cells.*

[0112] DNA constructs encoding the signal sequence, extracellular, transmembrane and intracellular domains of the parental HIV Gag TCR chains were synthesised (GeneArt, Germany). These TCR α chain and TCR β chain DNA sequences, provided in Figures 15a and 15b respectively, are altered from the parental HIV Gag TCR DNA sequences so as to enhance expression levels of the encoded TCR chains in human T cells whilst maintaing the native amino acid sequence. Figures 16a and 16b provide the full-length amino acid sequences encoded by the DNA sequences of Figures 15a and 15b respectively.

[0113] TCR α chain and TCR β chain DNA sequences were then inserted together into a Lentiviral expression vector. This vector contains DNA encoding both the parental HIV Gag TCR α chain and β chain as a single open reading frame with the in-frame

Foot and Mouth Disease Virus (FMDV) 2A cleaviage factor amino acid sequence (LLNFDLLKLAGDVESNPG (SEQ ID NO: 31)) separating the TCR chains. (de Felipe et al., Genet Vaccines Ther (2004) 2 (1): 13) On mRNA translation the TCR α chain is produced with the 2A peptide sequence at its C-terminus and the TCR β chain is produced as a separate polypeptide.

[0114] T cells were transduced with the above Lentiviral vector. Briefly, primary T cells were stimulated for 24 hours using anti-CD3/anti-CD28 beads. A concentrated Lentivirus supernatant, expressing the TCR genes, was then incubated with the stimulated T cells to allow viral transduction. The anti-CD3/anti-CD28 beads were then removed and the transduced T cells were cultured until they attained a "resting volume" of 200-300 fL.

[0115] Presentation of parental HIV Gag TCRs on the surface of the transduced cells was confirmed by FACS analysis using HLA-A*0201-SLYNTVALT PE tetramer and anti-CD8 monoclonal antibody FITC co-staining.

*Results*

[0116] Figure 17b provides the FACS analysis data which demonstrates the successful expression of the parental HIV Gag TCR on the surface of transduced $CD8^+$ T cells. Figure 17a provides FACS analysis data generated using control untransduced T cells.

*Example 7 - Inhibition of CTL activation by soluble high affinity HIV Gag TCRs*

[0117] The following assays were carried out to demonstrate that the soluble high affinity c11c6 HIV Gag TCR was capable of inhibiting activation of a SLYNTVATL-HLA-A*0201 reactive polyclonal T cell line.

*Inhibition of activation of the OX84 SLYNTVATL-HLA-A*0201 reactive polyclonal T cell line in the of presence of HIV infected cells*

[0118] The soluble c11c6 high affinity HIV Gag TCR utilised in this experiment contained the TCR alpha chain variable domain and TCR beta chain variable regions shown in Figure 6c (SEQ ID NO: 13) and Figure 7b (SEQ ID NO: 15) respectively. The full amino acid sequences of the TCR alpha and beta chains of this soluble TCR are provided by Figure 18a (SEQ ID NO: 29) and Figure 18b (SEQ ID NO: 30) respectively.

[0119] IFN-γ and TNF-α production was used as the read-outs for CTL activation.

*Reagents*

[0120] R10 Assay media: 10% FCS (heat-inactivated, Gibco, cat# 10108-165), 88% RPMI 1640 (Gibco, cat# 42401-018), 1% glutamine (Gibco, cat# 25030-024) and 1% penicillin/streptomycin (Gibco, cat# 15070-063).

[0121] Peptide: (obtained from various sources) initially dissolved in DMSO (Sigma, cat# D2650) at 4mg/ml and frozen.

[0122] The BD™ Cytometric Bead Array Kit, Human Th1/Th2 cytokine Kit II (BD Biosciences, San Diego, US) contains all the reagents required for the assay.

*T cell activation Assay*

[0123] Chronically HIV infected To target cells (HXB2 and HIV3B HIV Lab strains) were washed and re-suspended in R10 media. As a control uninfected To target cells were pulsed with 1nM of SLYNTVATL peptide, for 30 minutes at 37°C, 5%$CO_2$.

*Test Samples:*

**[0124]** 25,000 HIV infected To target cells in R10 media per well of a 96 well U-bottom plate.

**[0125]** 2 x 10$^{-7}$ M high affinity c11c6 HIV Gag TCR or parental HIV Gag TCR in R10 media per well.

**[0126]** 5000 OX84 polyclonal effector T cell line in R10 media per well.

***Controls:***

**[0127]** As above substituting irrelevant soluble TCRs (HLA-A*0201-Tax specific and HLA-A*0201-NY-ESO specific TCRs) or the high affinity HIV Gag TCRs.

**[0128]** The plate was then incubated for 4 hours at 37°C, 5% $CO_2$. The culture supernatant was removed to measure the levels of IFN-γ and TNF-α present using the following method.

***IFN-γ and TNF-α assay***

**[0129]** BD™ Cytometric Beads coated with (a) anti-IFNγ capture antibodies and (b) anti-TNFα capture antibodies were prepared according to the manufacturers instructions

**[0130]** A number of assay tubes were then prepared containing the following additions:

50 μl of mixed anti-IFNγ and anti-TNFα BD™ Cytometric Beads in BD Assay Diluent

50 μl of PE Dectection Reagent

**[0131]** Followed by either:

50 μl of the culture supernatant taken from the T cell activation assay wells. (Test Samples)
Or

50 μl of mixed IFNγ and TNFα standards prepared at a range of concentrations by serial dilution of stock standards. (Calibration Standards)

**[0132]** The tube were then incubated in the dark for 3 hours prior to being washed with ml of BD Wash Buffer and centrifuged. Finally, the beads were re-suspended in 300 μl of the Wash Buffer and the level of IFNγ and TNFα present was determined by Flow Cytometry according to manufacturer's instructions.

***Inhibition of the SLYNTVATL-HLA-A*0201 specific OX84 polyclonal T line in the presence of uninfected SLYNTVATL peptide pulsed To cells***

**[0133]** The same regents and methods as used for the above CTL activation assay were used except that:

2000 OX84 polyclonal effector T cells were used in each T cell activation assay.

**[0134]** Uninfected To lymphoblastoid cells, pulsed with 10$^{-10}$ -10$^{-8}$ M SLYNTVATL peptide were used as the target cells

***Results***

17

**[0135]** The soluble high affinity c11c6 HIV Gag TCR strongly inhibited activation of the SLYNTVATL-HLA-A*0201 reactive OX84 polyclonal T cell line in the presence of To cells infected by HIV as measured by IFN-γ and TNF-α production. (See Figure 19)

**[0136]** The soluble high affinity c11c6 HIV Gag TCR strongly inhibited activation of the SLYNTVATL-HLA-A*0201 reactive OX84 polyclonal T cell line in the presence of SLYNTVATL-pulsed uninfected To cells as measured by IFN-γ and TNF-α production. (See Figure 20)

***Example 8 - Quantification of cell surface SLYNTVATL-HLA-A*0201 antigens on peptide-pulsed T2 cells by fluorescence microscopy using high affinity c11c6 HIV Gag TCR***

**[0137]** The number of SLYNTVATL-HLA-A*0201 antigens on peptide-pulsed T2 lymphoblastoid cell was determined (on the assumption that one fluorescence signal relates to a single labelled TCR bound to its cognate pMHC ligand on the surface of the target cell) by single molecule fluorescence microscopy using a soluble high-affinity c11c6 HIV Gag TCR. This was facilitated by using biotinylated TCR to target the antigen-expressing cancer cells and subsequent labelling of cell-bound TCR by streptavidin-R phycoerythrin (PE) conjugates. Individual PE molecules were then imaged by 3-dimensional fluorescence microscopy.

**[0138]** T2 lymphoblastoid cells were pulsed with the HIV Gag-derived SLYNTVATL peptide, or an irrelevant peptide (SLLMWITQC) at a range of concentrations ($10^{-5}$ - $10^{-10}$M) for 90 minutes at 37°C. After pulsing the cells were washed twice with 500 µl of PBS. Cells were incubated in 200 µl of TCR solution (100 nM high-affinity c11c6 HIV Gag TCR), in PBS. 0.5% BSA albumin) for 30 min at room temperature. TCR solution was removed, and cells were washed three times with 500 µl of PBS. Cells were incubated in 200 µl of streptavidin-PE solution (5 µg ml$^{-1}$ streptavidin-PE in PBS containing 0.5% BSA) at room temperature in the dark for 20 min. Streptavidin-PE solution was removed and cells were washed three times with 500 µl of PBS. Wash media was removed, and cells kept in 400 µl of R10, without Phenol Red before imaging by fluorescence microscopy.

**[0139]** *Fluorescence microscopy.* Fluorescent microscopy was carried out using an Axiovert 200M (Zeiss) microscope with a 63x Oil objective (Zeiss). A Lambda LS light source containing a 300W Xenon Arc lamp (Sutter) was used for illumination, and light intensity was reduced to optimal levels by placing a 0.3 and a 0.6 neutral density filter into the light path. Excitation and emission spectra were separated using a TRITC/DiI filter set (Chroma). Cells were imaged in three dimensions by z-stack acquisition (21 planes, 1 µm apart). Image acquisition and analysis was performed using Metamorph software (Universal Imaging) as described (Irvine et al., Nature 419: p845-9, and Purbhoo et al., Nature Immunology 5: p524-30).

***Results***

**[0140]** As shown by Figure 21 the above method was used successfully to image high affinity c11c6 HIV Gag TCR bound to SLYNTVATL -HLA-A*0201 antigens on the surface of peptide-pulsed T2 cells. These results show the threshold for counting epitopes on SLYNTVATL peptide-pulsed cells using the high affinity c6c11 HIV Gag TCR is approxiamately $10^{-9}$ M peptide.

# REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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1

**PATENTKRAV**

1. T-celle-receptor (TCR), omfattende den variable region i TCR-alfa-kæden ifølge fig. 1a og den variable region i TCR-beta-kæden ifølge fig. 1b.

2. TCR ifølge krav 1, og som omfatter alfa-kæden ifølge fig. 1a og beta-kæden ifølge fig. 3b.

3. TCR ifølge krav 1, og som omfatter alfa-kæden ifølge fig. 5a og beta-kæden ifølge fig. 5b.

4. TCR ifølge et hvilket som helst af kravene 1 til 3, og som mangler trans-membran- og cytoplasmatiske områder.

5. Celle, som huser en TCR-ekspressionsvektor, der omfatter en nukleinsyre, som koder for TCR-alfa-kæden ifølge fig. 16a og betakæden i fig. 16b.

6. T-celle, omfattende en ekspressionsvektor, som omfatter en nukleinsyre, der koder for en TCR ifølge et hvilket som helst af kravene 1 til 3.

# DRAWINGS

**Figure 1a**

```
              10                    20
              *                     *
M  A  Q  K  E  V  E  Q  N  S  G  P  L  S  V  P  E  G  A  I  A  S  L  N  C  T  Y  S  D


30                    40                    50
*                     *                     *
R  G  S  Q  S  F  F  W  Y  R  Q  Y  S  G  K  S  P  E  L  I  M  F  I  Y  S  N  G  D  K

   60                    70                    80
   *                     *                     *
E  D  G  R  F  T  A  Q  L  N  K  A  S  Q  Y  I  S  L  L  I  R  D  S  K  L  S  D  S  A


   90                    100                   110
   *                     *                     *
T  Y  L  C  A  V  R  T  N  S  G  Y  A  L  N  F  G  K  G  T  S  L  L  V  T  P  H
```

(SEQ ID No: 1)

1

**Figure 1b**

```
        10                  20
         *                   *
M E A G V T Q S P T H L I K T R G Q Q V T L R C S P K S G


30                  40                  50
 *                   *                   *
H D T V S W Y Q Q A L G Q G P Q F I F Q Y Y E E E R Q R


    60                  70                  80
     *                   *                   *
G N F P D R F S G H Q F P N Y S S E L N V N A L L L G D S


       90                 100                 110
        *                   *                   *
A L Y L C A S S D T V S Y E Q Y F G P G T R L T V T
```
(SEQ ID NO: 2)

**Figure 2a**

atggcccagaaggaggtggagcagaattctggacccctcagtgttccagagggagccattgcctctctcaattgcacttaca
gtgaccgaggttcccagtccttcttctggtacagacaatattctgggaaaagccctgagttgataatgttcatatactccaatgg
tgacaaagaagatggaaggtttacagcacagctcaataaagccagccagtatatttccctgctcatcagagactccaagctc
agtgattcagccacctacctctgtgcggtgcgcacaaattccgggtatgcactcaacttcggcaaaggcacctcgctgttggt
cacaccccatatccagaaccctgaccctgccgtgtaccagctgagagactctaaatccagtgacaagtctgtctgcctattca
ccgattttgattctcaaacaaatgtgtcacaaagtaaggattctgatgtgtatatcacagacaaaactgtgctagacatgaggtc
tatggacttcaagagcaacagtgctgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaacaacagcatta
ttccagaagacaccttcttccccagcccagaaagttcctaa
(SEQ ID No: 3)

**Figure 2b**

atggaggctggagtcacacaaagtcccacacacctgatcaaaacgagaggacagcaagtgactctgagatgctctcctaa
gtctgggcatgacactgtgtcctggtaccaacaggccctgggtcaggggcccagtttatctttcagtattatgaggaggaag
agagacagagaggcaacttccctgatcgattctcaggtcaccagttccctaactatagctctgagctgaatgtgaacgccttg
ttgctgggggactcggccctctatctctgtgccagcagcgacaccgtctcctacgagcagtacttcgggccgggcaccagg
ctcacggtcacagaggacctgaaaaacgtgttcccacccgaggtcgctgtgtttgagccatcagaagcagagatctcccac
acccaaaaggccacactggtgtgcctggccacaggcttctaccccgaccacgtggagctgagctggtgggtgaatgggaa
ggaggtgcacagtggggtcagcacagacccgcagcccctcaaggagcagcccgccctcaatgactccagatacgctctg
agcagccgcctgagggtctcggccaccttctggcaggaccccgcaaccacttccgctgtcaagtccagttctacgggctc
tcggagaatgacgagtggacccaggatagggccaaacccgtcacccagatcgtcagcgccgaggcctggggtagagca
gactaa

(SEQ ID No: 4)

**Figure 3a**

```
M A Q K E V E Q N S G P L S V P E G A I A S L N C T Y S D R G S Q S F
F W Y R Q Y S G K S P E L I M F I Y S N G D K E D G R F T A Q L N K A
S Q Y I S L L I R D S K L S D S A T Y L C A V R T N S G Y A L N F G K
G T S L L V T P H I Q N P D P A V Y Q L R D S K S S D K S V C L F T D
F D S Q T N V S Q S K D S D V Y I T D K T V L D M R S M D F K S N S A
V A W S N K S D F A C A N A F N N S I I P E D T F F P S P E S S
```
(SEQ ID No: 5)

**Figure 3b**

```
M E A G V T Q S P T H L I K T R G Q Q V T L R C S P K S G H D T V S W
Y Q Q A L G Q G P Q F I F Q Y Y E E E E R Q R G N F P D R F S G H Q F
P N Y S S E L N V N A L L L G D S A L Y L C A S S D T V S Y E Q Y F G
P G T R L T V T E D L K N V F P P E V A V F E P S E A E I S H T Q K A
T L V C L A T G F Y P D H V E L S W W V N G K E V H S G V S T D P Q P
L K E Q P A L N D S R Y A L S S R L R V S A T F W Q D P R N H F R C Q
V Q F Y G L S E N D E W T Q D R A K P V T Q I V S A E A W G R A D
```
(SEQ ID No: 6)

4

**Figure 4a**

ccatcgatggcccagaaggaggtggagcagaattctggacccctcagtgttccagagggagccattgcctctctcaattgc
acttacagtgaccgaggttcccagtccttcttctggtacagacaatattctgggaaaagccctgagttgataatgttcatatactc
caatggtgacaaagaagatggaaggtttacagcacagctcaataaagccagccagtatatttccctgctcatcagagactcc
aagctcagtgattcagccacctacctctgtgcggtgcgcacaaattccgggtatgcactcaacttcggcaaaggcacctcgc
tgttggtcacaccccatatccagaaccctgaccctgccgtgtaccagctgagagactctaagtcgagtgacaagtctgtctgc
ctattcaccgatttтgattctcaaacaaatgtgtcacaaagtaaggattctgatgtgtatatcacagacaaatgtgtgctagacat
gaggtctatggacttcaagagcaacagtgctgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaacaac
agcattattccagaagacaccttcttccccagcccagaaagttcctaa
(SEQ ID No: 7)

**Figure 4b**

tctctcattaatggaggctggagtcacacaaagtcccacacacctgatcaaaacgagaggacagcaagtgactctgagatg
ctctcctaagtctgggcatgacactgtgtcctggtaccaacaggccctgggtcaggggccccagtttatctttcagtattatga
ggaggaagagagacagagaggcaacttccctgatcgattctcaggtcaccagttccctaactatagctctgagctgaatgtg
aacgccttgttgctggggggactcggccctctatctctgtgccagcagcgacaccgtctcctacgagcagtacttcgggccgg
gcaccagg
ctcacggtcacagaggacctgaaaaacgtgttcccaccccgaggtcgctgtgtttgagccatcagaagcagagatctcccac
acccaaaaggccacactggtgtgcctggccaccggtttctaccccgaccacgtggagctgagctggtgggtgaatgggaa
ggaggtgcacagtgggggtctgcacagacccgcagccctcaaggagcagcccgccctcaatgactccagatacgctctg
agcagccgcctgagggtctcggccaccttctggcaggacccccgcaaccacttccgctgtcaagtccagttctacgggctc
tcggagaatgacgagtggacccaggatagggccaaacccgtcacccagatcgtcagcgccgaggcctggggtagagca
gactaa
(SEQ ID No: 8)

**Figure 5a**

```
M A Q K E V E Q N S G P L S V P E G A I A S L N C T Y S D R G S Q S F
F W Y R Q Y S G K S P E L I M F I Y S N G D K E D G R F T A Q L N K A
S Q Y I S L L I R D S K L S D S A T Y L C A V R T N S G Y A L N F G K
G T S L L V T P H I Q N P D P A V Y Q L R D S K S S D K S V C L F T D
F D S Q T N V S Q S K D S D V Y I T D K C V L D M R S M D F K S N S A
V A W S N K S D F A C A N A F N N S I I P E D T F F P S P E S S
```
(SEQ ID No: 9)

**Figure 5b**

```
M E A G V T Q S P T H L I K T R G Q Q V T L R C S P K S G H D T V S W
Y Q Q A L G Q G P Q F I F Q Y Y E E E E R Q R G N F P D R F S G H Q F
P N Y S S E L N V N A L L L G D S A L Y L C A S S D T V S Y E Q Y F G
P G T R L T V T E D L K N V F P P E V A V F E P S E A E I S H T Q K A
T L V C L A T G F Y P D H V E L S W W V N G K E V H S G V C T D P Q P
L K E Q P A L N D S R Y A L S S R L R V S A T F W Q D P R N H F R C Q
V Q F Y G L S E N D E W T Q D R A K P V T Q I V S A E A W G R A D
```
(SEQ ID No: 10)

6

**Figure 6a**

M A Q K E V E Q N S G P L S V P E G A I A S L N C T Y S D R G S
Q S F F W Y R Q Y S G K S P E L I M F I Y S N G D K E D G R F T
A Q L N K A S Q Y I S L L I R D S K L S D S A T Y L C A V R <u>S</u> A
<u>H</u> G Y <u>S</u> L N F G K G T S L L V T P H
(SEQ ID NO: 11)

**Figure 6b**

M A Q K E V E Q N S G P L S V P E G A I A S L N C T Y S D R G S
Q S F F W Y R Q Y S G K S P E L I M F I Y S N G D K E D G R F T
A Q L N K A S Q Y I S L L I R D S K L S D S A T Y L C A V R <u>S</u> A
<u>H</u> G Y A L N F G K G T S L L V T P H
(SEQ ID NO: 12)

**Figure 6c**

M A Q K E V E Q N S G P L S V P E G A I A S L N C T Y S D R G S
Q S F F W Y R Q Y S G K S P E L I M F I Y S N G D K E D G R F T
A Q L N K A S Q Y I S L L I R D S K L S D S A T Y L C A V R <u>G</u> A
<u>H</u> <u>D</u> Y A L N F G K G T S L L V T P H
(SEQ ID NO: 13)

7

**Figure 7a**

M E A G V T Q S P T H L I K T R G Q Q V T L R C S P K S G H D T
V S W Y Q Q A L G Q G P Q F I F Q Y <u>V R G V</u> E R Q R G N F P D R
F S G H Q F P N Y S S E L N V N A L L L G D S A L Y L C A S S D
T V S Y E Q Y F G P G T R L T V T
(SEQ ID NO: 14)

**Figure 7b**

M E A G V T Q S P T H L I K T R G Q Q V T L R C S P K S G H D T
V S W Y Q Q A L G Q G P Q F I F Q Y <u>A L G</u> E E R Q R G N F P D R
F S G H Q F P N Y S S E L N V N A L L L G D S A L Y L C A S S D
T V S Y E Q Y F G P G T R L T V T
(SEQ ID NO: 15)

**Figure 8a**

```
I  Q  N  P  D  P  A  V  Y  Q  L  R  D  S  K  S  S  D  K  S  V  C  L  F  T
D  F  D  S  Q  T  N  V  S  Q  S  K  D  S  D  V  Y  I  T  D  K
```
(SEQ ID NO: 19)

**Figure 8b**

```
E  D  L  N  K  V  F  P  P  E  V  A  V  F  E  P  S  E  A  E  I  S  H  T  Q  K  A  T
L  V  C  L  A  T  G  F  F  P  D  H  V  E  L  S  W  W  V  N  G  K  E  V  H  S  G  V
```
(SEQ ID NO: 20)

**Figure 8c**

```
E  D  L  K  N  V  F  P  P  E  V  A  V  F  E  P  S  E  A  E  I  S  H  T  Q  K  A  T
L  V  C  L  A  T  G  F  Y  P  D  H  V  E  L  S  W  W  V  N  G  K  E  V  H  S  G  V
```
(SEQ ID NO: 21)

9

Figure 9

**PEX954**

gatctcgatcccgcgaaattaatacgactcactatagggagaccacaacggtttccctctagaaataattttgtttaactttaaga

aggagatataatcgatgtctaactcgagtgacaagtctgtctgcctattcaccgattttgattctcaaacaaatgtgtcacaaagt

aaggattctgatgtgtatatcacagacaaatgtgtgctagacatgaggtctatggacttcaagagcaacagtgctgtggcctg

gagcaacaaatctgactttgcatgtgcaaacgccttcaacaacagcattattccagaagacaccttcttccccagcccagaaa

gttcctaagcttgaattccgatccggctgctaacaaagcccgaaaggaagctgagttggctgctgccaccgctgagcaataa

ctagcataaccccttggggcctctaaacgggtcttgaggggttttttgctgaaaggaggaactatatccggataattcttgaag

acgaaagggcctcgtgatacgcctattttataggttaatgtcatgataataatggtttcttagacgtgaggtggcactttcggg

gaaatgtgcgcggaacccctatttgtttattttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgc

ttcaataatattttgttaaaattcgcgttaaattttgttaaatcagctcattttttaaccaataggccgaaatcggcaaaatcccttat

aaatcaaaagaatagaccgagatagggttgagtgttgttccagtttggaacaagagtccactattaaagaacgtggactccaa

cgtcaaagggcgaaaaaccgtctatcagggcgatggcccactacgtgaaccatcaccctaatcaagtttttttggggtcgagg

tgccgtaaagcactaaatcggaaccctaaagggagcccccgatttagagcttgacggggaaagccggcgaacgtggcga

gaaaggaagggaagaaagcgaaaggagcgggcgctagggcgctggcaagtgtagcggtcacgctgcgcgtaaccacc

acacccgccgcgcttaatgcgccgctacagggcgcgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtt

tattttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagta

tgagtattcaacatttccgtgtcgcccttattcccttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaa

gtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttt

tcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtgttgacgccgggca

agagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatg

gcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcgg

aggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaat

gaagccataccaaacgacgagcgtgacaccacgatgcctgcagcaatggcaacaacgttgcgcaaactattaactggcga

actacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggccc

ttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagat

ggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgag

Figure 9 Cont.

ataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaattt

aaaaggatctaggtgaagatccttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagacc

ccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctacc

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ctgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgtt

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(SEQ ID NO: 22)

11

Figure 10

**PEX821**

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**Figure 10 Cont.**

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(SEQ ID NO: 23)

13

Figure 11

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P N Y S S E L N V N A L L L G D S A L Y L C A S S D T V S Y E Q Y F G
P G T R L T V T E D L K N V F P P E V A V F E P S E A E I S H T Q K A
T L V C L A T G F Y P D H V E L S W W V N G K E V H S G V C T D P Q P
L K E Q P A L N D S R Y A L S S R L R V S A T F W Q D P R N H F R C Q
V Q F Y G L S E N D E W T Q D R A K P V T Q I V S A E A W G R A D P G
A P T S S S T K K T Q L Q L E H L L L D L Q M I L N G I N N Y K N P K
L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L
N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C
E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
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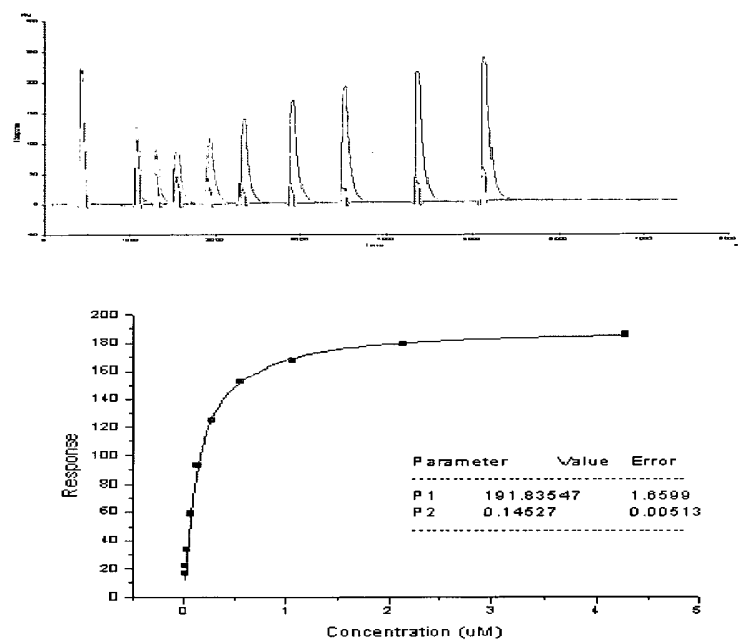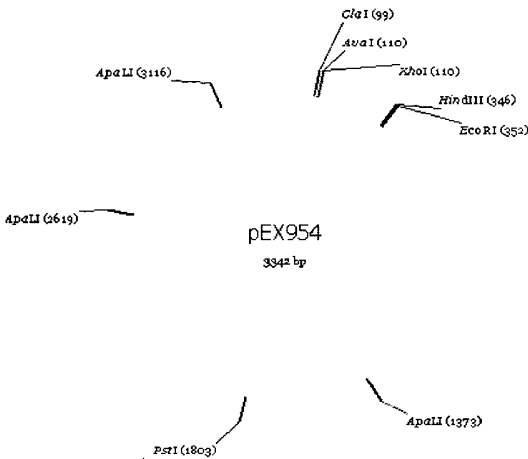(SEQ ID NO: 24)

14

Figure 12

Figure 13



ClaI (99)
AvaI (110)
XhoI (110)
HindIII (346)
EcoRI (352)
ApaLI (3116)
ApaLI (2619)

pEX954
3342 bp

PstI (1803)
ApaLI (1373)

Figure 14



NdeI (99)
PstI (168)
ApaLI (3610)
AvaI (473)
AgeI (548)
ApaLI (602)
ApaLI (3113)
HindIII (840)
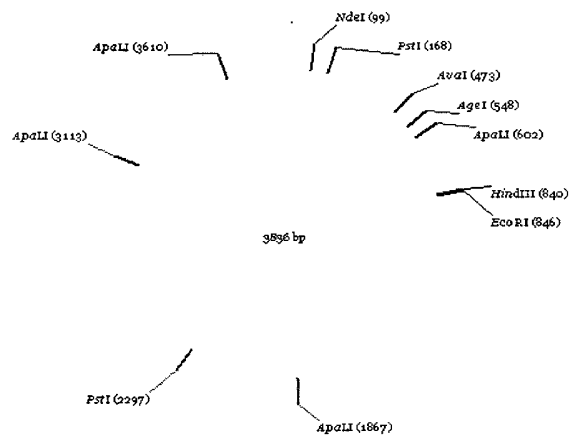EcoRI (846)
3836 bp
PstI (2297)
ApaLI (1867)

**Figure 15a**

```
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(SEQ ID NO: 25)
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**Figure 15b**

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(SEQ ID NO: 26)
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18

**Figure 16a**

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M M K S L R V L L V I L W L Q L S W V W S Q Q K E V E Q N S G P L S V
P E G A I A S L N C T Y S D R G S Q S F F W Y R Q Y S G K S P E L I M
F I Y S N G D K E D G R F T A Q L N K A S Q Y I S L L I R D S K L S D
S A T Y L C A V R T N S G Y A L N F G K G T S L L V T P H I Q N P D P
A V Y Q L R D S K S S D K S V C L F T D F D S Q T N V S Q S K D S D V
Y I T D K T V L D M R S M D F K S N S A V A W S N K S D F A C A N A F
N N S I I P E D T F F P S P E S S C D V K L V E K S F E T D T N L N F
Q N L S V I G F R I L L L K V A G F N L L M T L R L W S S
```
(SEQ ID NO: 27)

**Figure 16b**

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M G P G L L C W A L L C L L G A G L V D A G V T Q S P T H L I K T R G
Q Q V T L R C S P K S G H D T V S W Y Q Q A L G Q G P Q F I F Q Y Y E
E E E R Q R G N F P D R F S G H Q F P N Y S S E L N V N A L L L G D S
A L Y L C A S S D T V S Y E Q Y F G P G T R L T V T E D L K N V F P P
E V A V F E P S E A E I S H T Q K A T L V C L A T G F Y P D H V E L S
W W V N G K E V H S G V S T D P Q P L K E Q P A L N D S R Y C L S S R
L R V S A T F W Q N P R N H F R C Q V Q F Y G L S E N D E W T Q D R A
K P V T Q I V S A E A W G R A D C G F T S E S Y Q Q G V L S A T I L Y
E I L L G K A T L Y A V L V S A L V L M A M V K R K D S R G
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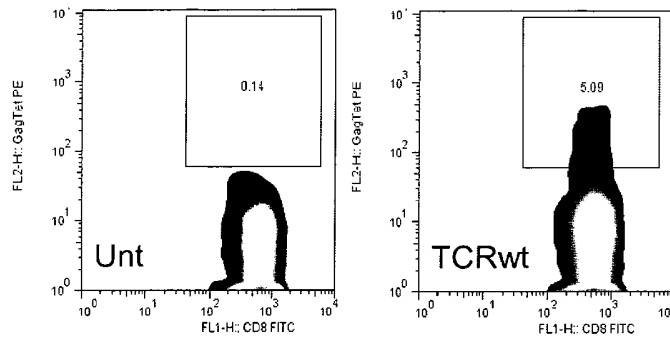(SEQ ID NO: 28)

Figure 17



**Figure 17a**        **Figure 17b**

**Figure 18a**

M A Q K E V E Q N S G P L S V P E G A I A S L N C T Y S D R G S

Q S F F W Y R Q Y S G K S P E L I M F I Y S N G D K E D G R F T

A Q L N K A S Q Y I S L L I R D S K L S D S A T Y L C A V R <u>G A</u>

<u>H D</u> Y A L N F G K G T S L L V T P H <u>I</u> Q N P D P A V Y Q L R D S

K S S D K S V C L F T D F D S Q T N V S Q S K D S D V Y I T D K

C V L D M R S M D F K S N S A V A W S N K S D F A C A N A F N N

S I I P E D T F F P S P E S S

(SEQ ID NO: 29)

**Figure 18b**

M E A G V T Q S P T H L I K T R G Q Q V T L R C S P K S G H D T

V S W Y Q Q A L G Q G P Q F I F Q Y <u>A L G</u> E E R Q R G N F P D R

F S G H Q F P N Y S S E L N V N A L L L G D S A L Y L C A S S D

T V S Y E Q Y F G P G T R L T V T E D L K N V F P P E V A V F E

P S E A E I S H T Q K A T L V C L A T G F Y P D H V E L S W W V

N G K E V H S G V C T D P Q P L K E Q P A L N D S R Y A L S S R

L R V S A T F W Q D P R N H F R C Q V Q F Y G L S E N D E W T Q

D R A K P V T Q I V S A E A W G R A D

(SEQ ID NO: 30)

Figure 19

Figure 20

Figure 21