

T CELL RECEPTORS SPECIFIC FOR THE NY-ESO-1 TUMOR ANTIGEN-HLA-A*02 COMPLEX

5 The present invention relates to T cell receptors (TCRs) which bind the HLA-A*02 restricted peptide SLLMWITQC derived from the cancer antigen NY-ESO-1. Said TCRs may comprise mutations within the alpha and/or beta variable domains relative to a native NY-ESO-1 TCR. The TCRs of the invention are particularly suitable for use as novel immunotherapeutic reagents for the treatment of malignant disease.

10 **Background to the invention**

T cell receptors (TCRs) are naturally expressed by CD4⁺ and CD8⁺ T cells. TCRs are designed to recognize short peptide antigens that are displayed on the surface of antigen presenting cells in complex with Major Histocompatibility Complex (MHC) molecules (in humans, MHC molecules are also known as Human Leukocyte Antigens, or HLA) (Davis, et al., (1998), Annu Rev Immunol 16: 523-544.). CD8⁺ T cells, which are also termed cytotoxic T cells, specifically recognize peptides bound to MHC class I and are generally responsible for finding and mediating the destruction of infected or cancerous cells.

20 NY-ESO-1 belongs to the family of germline encoded cancer antigens (Chen, et al., (1997), Cytogenet Cell Genet 79(3-4): 237-240: WO9814464) and has the Uniprot accession number P78358. Such germline antigens have been found to be frequently expressed in a variety of cancers, while their expression in normal tissues is limited to adult testes and other immune privileged sites. The cancer specific nature of these antigens makes them ideal targets for anti-cancer therapeutics. The precise function of NY-ESO-1 remains unknown but it's expression has been reported in foetal and adult testes (Satie, et al., (2002), Lab Invest 82(6): 775-780), ovary and uterine myometrium, as well as in a wide variety of cancers including myeloma (Andrade, et al., (2008), Cancer Immun 8: 2), ovarian cancer (Odunsi, et al., (2003), Cancer Res 63(18): 6076-6083), non-small cell lung cancer (Konishi, et al., (2004), Oncol Rep 11(5): 1063-1067) and melanoma (Barrow, et al., (2006), Clin Cancer Res 12(3 Pt 1): 764-771). The 9-mer peptide SLLMWITQC (SEQ ID NO 1) corresponds to amino acids 157–165 of the full length NY-ESO-1 protein. The same peptide is also found in LAGE-1A (Acc. No. O75638-2), an alternative cancer antigen (Lethe, et al., (1998), Int J Cancer 76(6): 903-908). This peptide binds to HLA-A*02 and the peptide-HLA complex is able to stimulate cytotoxic T cells leading to lysis of NY-ESO-1⁺, HLA-A*02⁺, tumour cells (Duffour, et al., (1999), Eur J Immunol 29(10): 3329-3337 and WO2000020445). The SLLMWITQC HLA-A*02 complex therefore provides a useful target antigen for immunotherapeutic intervention.

The identification of particular TCR sequences that bind to SLLMWITQC HLA-A*02 complex is advantageous for the development of novel immunotherapies. Such therapeutic TCRs may be used, for example, as soluble targeting agents for the purpose of delivering cytotoxic or immune effector agents to the tumour (Lissin, et al., (2013). "High-Affinity Monoclonal T-cell receptor (mTCR) Fusions. Fusion Protein Technologies for Biopharmaceuticals: Applications and Challenges". S. R. Schmidt, Wiley; Boulter, et al., (2003), Protein Eng 16(9): 707-711; Liddy, et al., (2012), Nat Med 8: 980-987), or alternatively they may be used to engineer T cells for adoptive therapy (June, et al., (2014), Cancer Immunol Immunother 63(9): 969-975). It is desirable that TCRs for immunotherapeutic use are able to strongly recognize the target antigen, by which it is meant that the TCR should possess a high affinity and / or long binding half-life for the target antigen in order to exert a potent response. TCRs as they exist in nature typically have low affinity for target antigen (low micromolar range), thus it is often necessary to identify mutations, including but not limited to substitutions, insertions and/or deletions, that can be made to a given TCR sequence in order to improve antigen binding. For use as soluble targeting agents TCR antigen binding affinities in the nanomolar to picomolar range and with binding half-lives of several hours are preferable. It is also desirable that therapeutic TCRs demonstrate a high level of specificity for the target antigen to mitigate the risk of toxicity in clinical applications resulting from off-target binding. Such high specificity may be especially challenging to obtain given the natural degeneracy of TCR antigen recognition (Wooldridge, et al., (2012), J Biol Chem 287(2): 1168-1177; Wilson, et al., (2004), Mol Immunol 40(14-15): 1047-1055). Finally, it is desirable that therapeutic TCRs are able to be expressed and purified in a highly stable form.

The TCR sequences defined herein are described with reference to IMGT nomenclature which is widely known and accessible to those working in the TCR field. For example, see: LeFranc and LeFranc, (2001). "T cell Receptor Factsbook", Academic Press; Lefranc, (2011), Cold Spring Harb Protoc 2011(6): 595-603; Lefranc, (2001), Curr Protoc Immunol Appendix 1: Appendix 1O; and Lefranc, (2003), Leukemia 17(1): 260-266. $\alpha\beta$ TCRs consist of two disulphide linked chains. Each chain (alpha and beta) is generally regarded as having two domains, namely a variable and a constant domain. A short joining region connects the variable and constant domains and is typically considered part of the variable region. Additionally, the beta chain usually contains a short diversity region between the variable and joining regions.

The variable domain of each chain is located N-terminally and comprises three Complementarity Determining Regions (CDRs) embedded in a framework sequence. The CDRs comprise the recognition site for peptide-MHC binding. There are several genes coding for alpha chain variable ($V\alpha$) regions and several genes coding for beta chain variable ($V\beta$) regions. These genes are distinguished by their framework, CDR1 and CDR2 sequences, and by a partly defined CDR3 sequence. The $V\alpha$ and $V\beta$ genes are referred to in IMGT nomenclature by the prefixes 'TRAV' and 'TRBV' respectively (Folch and Lefranc, (2000), Exp Clin Immunogenet 17(1): 42-54; Scaviner and

Lefranc, (2000), Exp Clin Immunogenet 17(2): 83-96; LeFranc and LeFranc, (2001), "T cell Receptor Factsbook", Academic Press). Likewise there are several joining or J genes, termed 'TRAJ' or 'TRBJ', for the alpha and beta chain respectively, and for the beta chain, a diversity or D gene termed 'TRBD' (Folch and Lefranc, (2000), Exp Clin Immunogenet 17(2): 107-114; Scaviner and Lefranc, (2000), Exp Clin Immunogenet 17(2): 97-106; LeFranc and LeFranc, (2001), "T cell Receptor Factsbook", Academic Press). The huge diversity of alpha and beta variable region sequences results from combinatorial rearrangements between the various V, J and D genes, which include allelic variants, and additional junctional diversity (Arstila, et al., (1999), Science 286(5441): 958-961; Robins et al., (2009), Blood 114(19): 4099-4107.) The constant, or C, regions of TCR alpha and beta chains are referred to as 'TRAC' and 'TRBC' respectively (Lefranc, (2001), Curr Protoc Immunol Appendix 1: Appendix 1O).

In the present specification and claims, the term "TCR alpha (or α) variable domain" refers to the concatenation of TRAV and TRAJ regions; a TRAV region only; or TRAV and a partial TRAJ region, and the term TCR alpha (or α) constant domain refers to the extracellular TRAC region, or to a C-terminal truncated or full length TRAC sequence. Likewise the term "TCR beta (or β) variable domain" may refer to the concatenation of TRBV and TRBD/TRBJ regions; to the TRBV and TRBD regions only; to the TRBV and TRBJ regions only; or to the TRBV and partial TRBD and/or TRBJ regions, and the term TCR beta (or β) constant domain refers to the extracellular TRBC region, or to a C-terminal truncated or full length TRBC sequence.

TCRs that target NY-ESO-1 have been previously reported (WO05113595; WO08039818; McCormack, et al., (2013), Cancer Immunol Immunother 62(4): 773-785).

The inventors have identified alternative TCR alpha and beta variable domain sequences that bind to the NY-ESO-1 HLA-A*02 complex. Such sequences are particularly suitable for use as therapeutic TCRs for targeted immunotherapy of cancers that present SLLMWITQC HLA-A*02 complex.

The inventors have identified a native TCR comprising the following chain usage:

Alpha chain: TRAV3*01/TRAJ28*01

Beta chain: TRBV29-1*01/TRBD2*01/TRBJ2-3*01

(Note, the term '*01' indicates the allelic variant for this sequence, as designated by IMGT nomenclature)

This native TCR was used as a template from which the mutated sequences of the invention were derived.

Summary of Invention

The present invention provides as a first aspect a T cell receptor (TCR) having the property of binding to SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex and comprising a TCR alpha chain

- 5 variable domain and/or a TCR beta chain variable domain, wherein

the alpha chain variable domain comprises an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to amino acids 1-117 of SEQ ID NO: 2, and/or

- 10 the beta chain variable domain comprises an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to amino acids 1-115 of SEQ ID NO: 3.

As a second aspect, the invention provides a TCR that binds to a SLLMWITQC (SEQ ID NO: 1)

- 15 HLA-A*02 complex with an affinity greater than 50 μ M, wherein: the alpha chain CDRs 1, 2 and 3 comprise SEQ ID NO: 41, 42 and 43 respectively, and/or the beta chain CDRs 1, 2 and 3 comprise SEQ ID NO: 44, 45 and 46 respectively; and/or at least one of the CDRs contains one or more conservative substitutions with respect to SEQ ID NO: 41 to 46; and/or at least one of the CDRs contains up to three tolerated substitutions with respect to SEQ ID NO: 41 to 46.

- 20 The alpha chain variable domain of the first or second aspect may have at least one of the following mutations with reference to the numbering of SEQ ID NO: 2:

Residue no.	
I51	L
T52	G
G53	D
D54	S
N55	A
D95	S
I96	S
N97	D or R
S98	Q
G99	H

and/or the beta chain variable domain of the first or second aspect may have at least one of the

- 25 following mutations with reference to the numbering of SEQ ID NO: 3:

Residue no.	
S27	K
Q28	R
V29	L

T30	A
M31	L
N50	W
Q51	T
S53	G
G100	A

The alpha chain variable domain may have at least one of the following mutations with reference to the numbering of SEQ ID NO: 2:

Residue no.	
I51	L
G53	D

- 5 And/or the beta chain variable domain may have at least one of the following mutations with reference to the numbering of SEQ ID NO: 3:

Residue no.	
T30	A
N50	W
G100	A

- 10 The alpha chain variable domain may have between 2 and 6 of the following mutations with reference to the numbering of SEQ ID NO: 2:

Residue no.	
I51	L
T52	G
G53	D
D54	S
N55	A
D95	S
I96	S
N97	D or R
S98	Q
G99	H

and / or the beta chain variable domain may have between 3 and 9 of the following mutations with reference to SEQ ID NO: 3:

- 15

Residue no.	
S27	K
Q28	R

V29	L
T30	A
M31	L
N50	W
Q51	T
S53	G
G100	A

The alpha chain variable domain may have at least one of the following groups of mutations

Group 1: I51L, T52G, G53D, D54S, N55A

Group 2: I51L, T52G, G53D, D54S, N55A, N97D

5 Group 3: I51L, T52G, G53D, D54S, N55A, N97R

Group 4: I96S, N97D, S98Q, G99H

Group 5: D95S, I96S, N97R, S98Q, G99H

Group 6: I51L, G53D

and/or the beta chain variable domain may have at least one of the following groups of mutations

10 Group 1: N50W, Q51T, S53G

Group 2: S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G

Group 3: S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G, G100A

Group 4: S27K, Q28R, V29L, T30A, N50W, Q51T

Group 5: S27K, Q28R, V29L, T30A, N50W, Q51T S53G

15 Group 6: S27K, Q28R, V29L, T30A, N50W, Q51T S53G, G100A

Group 7: S27K, Q28R, V29L, T30A, M31L, N50W, Q51T

Group 8: T30A, N50W, G100A

20 The alpha chain variable domain and beta chain variable domain may have the following groups of mutations, respectively:

alpha chain		beta chain	
Group 1	I51L, T52G, G53D, D54S, N55A	Group 3	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G, G100A
Group 1	I51L, T52G, G53D, D54S, N55A	Group 7	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T
Group 3	I51L, T52G, G53D, D54S, N55A, N97R	Group 3	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G, G100A
Group 3	I51L, T52G, G53D, D54S, N55A, N97R	Group 7	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T

The TCR of the invention may comprise an alpha chain variable domain that has the following mutation with reference to the numbering of SEQ ID NO: 2:

Residue no.	
Q10	L

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and/or the beta chain variable domain may have at least one of the following mutations with reference to the numbering of SEQ ID NO: 3:

Residue no.	
C13	K
L43	P

- 10 In the alpha chain variable domain the sequence of amino acid residues 27 - 32, 50 - 57 and 91 - 107 is selected from the following:

Residues 27 - 32	Residues 50 - 57	Residues 91 - 107
VSGNPY	YITGDNLV	CAVRDSDQHAGSYQLTF
VSGNPY	YLGDSALV	CAVRDINSAGSYQLTF
VSGNPY	YITGDNLV	CAVRSSRQHAGSYQLTF
VSGNPY	YLGDSALV	CAVRDIDSGAGSYQLTF
VSGNPY	YLGDSALV	CAVRDIRSGAGSYQLTF
VSGNPY	YLTDDNLV	CAVRDINSAGSYQLTF

- 15 The TCR alpha chain variable domain may comprise an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to SEQ ID NO: 12.

In the beta chain variable domain the sequence of amino acid residues 27 - 31, 49 - 55 and 93 - 106 is selected from the following:

Residues 27 - 31	Residues 49 - 55	Residues 93 - 106
KRLAL	AWTGGEA	CSVGGSGGADTQYF
KRLAL	AWTGGEA	CSVGGSGAADTQYF
KRLAM	AWTGGEA	CSVGGSGGADTQYF
KRLAL	AWTGSEA	CSVGGSGGADTQYF
KRLAM	AWTGGEA	CSVGGSGAADTQYF
KRLAM	AWTGSEA	CSVGGSGGADTQYF
SQVTM	AWTGGEA	CSVGGSGGADTQYF

SQVAM	AWQGSEA	CSVGGSGAADTQYF
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The TCR beta chain variable domain may comprise an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to SEQ ID NO: 15.

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The alpha chain variable domain may have the sequence of amino acids residues 27-32, 50-57 and 91-107 and the beta chain variable domain may have the sequence of amino acid residues 27-31, 49-55 and 93-106 selected from the following:

Alpha chain			Beta Chain		
27-32	50-57	91-107	27-31	49-55	93-106
VSGNPY	YLGDSALV	CAVRDINSAGAGSYQLTF	KRLAL	AWTGGEA	CSVGGSGAADTQYF
VSGNPY	YLGDSALV	CAVRDINSAGAGSYQLTF	KRLAL	AWTGSEA	CSVGGSGGADTQYF
VSGNPY	YLGDSALV	CAVRDIRSGAGSYQLTF	KRLAL	AWTGGEA	CSVGGSGAADTQYF
VSGNPY	YLGDSALV	CAVRDIRSGAGSYQLTF	KRLAL	AWTGSEA	CSVGGSGGADTQYF

10

The alpha chain variable domain may comprise an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 100% identity to SEQ ID NO: 12 and the beta chain variable domain may comprise an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 15.

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The alpha chain variable domain may be selected from the amino acid sequence of SEQ ID NO: 6-12, or 51 and the beta chain variable domain may be selected from the amino acid sequence of SEQ ID NO: 13-23, or 52.

The alpha chain variable domain and the beta chain variable domain may be selected from the amino acid sequences of:

20

Alpha chain variable domain	Beta chain variable domain
SEQ ID NO: 7	SEQ ID NO: 15
SEQ ID NO: 7	SEQ ID NO: 18
SEQ ID NO: 12	SEQ ID NO: 15
SEQ ID NO: 12	SEQ ID NO: 18

The TCR of the invention may be an alpha-beta heterodimer, having an alpha chain TRAC constant domain sequence and a beta chain TRBC1 or TRBC2 constant domain sequence.

- The alpha and beta chain constant domain sequences may be modified by truncation or substitution to delete the native disulphide bond between Cys4 of exon 2 of TRAC and Cys2 of exon 2 of TRBC1 or TRBC2 and/or the alpha and/or beta chain constant domain sequence(s) may be modified by substitution of cysteine residues for Thr 48 of TRAC and Ser 57 of TRBC1 or
- 5 TRBC2, the said cysteines forming a non-native disulphide bond between the alpha and beta constant domains of the TCR.

The TCR of the invention may be in single chain format of the type $V\alpha$ -L- $V\beta$, $V\beta$ -L- $V\alpha$, $V\alpha$ -C α -L- $V\beta$, $V\alpha$ -L- $V\beta$ -C β , wherein $V\alpha$ and $V\beta$ are TCR α and β variable regions respectively, C α and C β are TCR α and β constant regions respectively, and L is a linker sequence.

- 10 The TCR of the invention may be associated with a detectable label, a therapeutic agent or a PK modifying moiety.

- The invention also provides a TCR anti-CD3 fusion comprising the TCR of the invention and an anti-CD3 antibody covalently linked to the C- or N-terminus of the alpha or beta chain of the TCR and such a TCR anti-CD3 fusion may comprise an alpha chain variable domain selected from any
- 15 one of SEQ ID NO: 6-12, or 51 and a beta chain variable domain selected from any one of SEQ ID NO: 13-23, or 52. The beta chain may be linked to the anti-CD3 antibody sequence via a linker sequence; the linker sequence may be selected from the group consisting of GGGGS (SEQ ID NO: 24), GGGSG (SEQ ID NO: 25), GGSGG (SEQ ID NO: 26), GSGGG (SEQ ID NO: 27), GSGGGP (SEQ ID NO: 28), GGEPS (SEQ ID NO: 29), GGEGGGP (SEQ ID NO: 30), and GGEGGGSEGGGS
- 20 (SEQ ID NO: 31).

The TCR anti-CD3 fusion of the invention may comprise an alpha chain amino acid sequence and beta chain amino acid sequence pairing that have at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to the amino acid sequence pairings as set forth in the table below.

	alpha chain SEQ ID NO:	beta chain SEQ ID NO:	Shown in
ImmTAC1	32	33	Figure 5
ImmTAC2	35	36	Figure 6
ImmTAC3	37	38	Figure 7
ImmTAC4	39	40	Figure 8

25

The invention also provides a nucleic acid encoding a TCR or the TCR anti-CD3 fusion of the invention.

- Also provided is a non-naturally occurring and/or purified and/or engineered cell presenting a TCR or TCR anti-CD3 fusion of the invention a cell harbouring (a) a TCR expression vector which
- 30 comprises nucleic acid of the invention in a single open reading frame, or two distinct open reading

frames encoding the alpha chain and the beta chain respectively; or (b) a first expression vector which comprises nucleic acid encoding the alpha chain of a TCR or TCR anti-CD3 fusion of the invention, and a second expression vector which comprises nucleic acid encoding the beta chain of a TCR or TCR anti-CD3 fusion of the invention.

- 5 The cell presenting the TCR or TCR anti-CD3 fusion or the cell harbouring the expression vector(s) may be a T-cell.

The invention also provides a pharmaceutical composition comprising a TCR or TCR anti-CD3 fusion of the invention or a cell of the invention, together with one or more pharmaceutically acceptable carriers or excipients.

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The TCR, TCR anti-CD3 fusion or a nucleic acid or a cell of the invention for use in medicine, is also provided. The TCR, TCR anti-CD3 fusion, cell or nucleic acid may be for use in a method of treating cancer in a human subject.

- 15 The human subject may have a tumour that expresses NY-ESO-1 and/or LAGE-1A and the tumour may be a solid tumour and be selected from a synovial sarcoma, non small cell lung carcinoma (NSCLC), bladder tumour, gastric tumour, prostate tumour, colorectal tumour, breast tumour, ovarian tumour, oesophageal tumour, melanoma, multiple myeloma, hepatocellular carcinoma and head and neck tumour. The human subject may be a subject of HLA-A*02 subtype.

20

Detailed description of the invention

- In a first aspect the invention provides a T cell receptor (TCR) having the property of binding to SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex and comprising a TCR alpha chain variable domain and/or a TCR beta chain variable domain, wherein the alpha chain variable domain
- 25 comprises an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to amino acids 1-117 of SEQ ID NO: 2, and/or the beta chain variable domain comprises an amino acid sequence that has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to amino acids 1-115 of SEQ ID NO: 3. The TCR may be isolated, cell free and/or soluble, i.e. it may not be a
- 30 TCR that occurs in its natural state within a T-cell within a human body.

- The invention also provides a TCR that binds to a SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex with an affinity greater than 50 μ M, wherein: the alpha chain CDRs 1, 2 and 3 comprise SEQ ID NO: 41, 42 and 43 respectively, and/or the beta chain CDRs 1, 2 and 3 comprise SEQ ID
- 35 NO: 44, 45 and 46 respectively; and/or at least one of the CDRs contains one or more conservative substitutions with respect to SEQ ID NO: 41 to 46; and/or at least one of the CDRs contains up to

three tolerated substitutions with respect to SEQ ID NO: 41 to 46. The affinity of the TCRs for the SLLMWITQC HLA-A*02 complex may be in the range 50 μ M to 100 nM. Preferably said substitutions do not change the binding affinity by more than $\pm$ 50%, or more preferably by no more than $\pm$ 20%, relative the non-substituted TCR.

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For the production of stable, soluble TCRs of the invention, a soluble version of the native TCR was used as starting sequence having the sequence shown in Figure 2. For this purpose cysteine substitutions are introduced into the TRAC and TRBC regions such that a non-native interchain disulphide bond can be formed. Suitable positions for the location of said cysteine substitutions are described in WO03020763. Figure 2 shows the extracellular sequences of the wild type TCR alpha and beta chains respectively, in soluble format. SEQ ID NO: 4 is identical to the native alpha chain extracellular sequence SEQ ID NO: 2 except that Thr48 of TRAC has been replaced with Cys. Likewise SEQ ID NO: 5 is identical to the native beta chain extracellular sequence SEQ ID NO: 3 except that Ser57 of TRBC has been replaced with Cys, Cys75 has been replaced with Ala and Asn201 has been replaced with Asp. The soluble wild-type TCR described above may be used to provide a reference against which the binding profile of mutated TCRs of the invention may be compared. A TCR of the first aspect may also be a TCR of the second aspect.

TCRs of either or both aspects the invention may be non-naturally occurring and/or purified and/or engineered. TCRs of the invention may have more than one mutation present in the alpha chain variable domain and/or the beta chain variable domain relative to the native NY-ESO-1 TCR.

"Engineered TCR" and "mutant TCR" are used synonymously herein and generally mean a TCR which has one or more mutations introduced relative to the wild-type NY-ESO-1 TCR, in particular in the alpha chain variable domain and/or the beta chain variable domain thereof. Mutations are preferably made within the CDR regions. These mutation(s) typically improve the binding affinity of the TCR to the SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex. The alpha chain variable domain may have at least one, two, three, four, five or six of the following mutations with reference to the numbering of SEQ ID NO: 2:

30

Residue no.	
I51	L
T52	G
G53	D
D54	S
N55	A
D95	S
I96	S
N97	D or R
S98	Q

G99	H
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(table 1)

and/or the beta chain variable domain may have at least one, two, three, four, five, six, seven, eight or nine of the following mutations with reference to the numbering of SEQ ID NO: 3:

5

Residue no.	
S27	K
Q28	R
V29	L
T30	A
M31	L
N50	W
Q51	T
S53	G
G100	A

(table 2)

The alpha chain variable domain may have at least one of the following mutations with reference to the numbering of SEQ ID NO: 2:

10

Residue no.	
I51	L
G53	D

(table 3)

And/or the beta chain variable domain may have at least one of the following mutations with reference to the numbering of SEQ ID NO: 3:

15

Residue no.	
T30	A
N50	W
G100	A

(table 4)

The alpha chain variable domain has at least one of the following groups of mutations

Group 1: I51L, T52G, G53D, D54S, N55A

20 Group 2: I51L, T52G, G53D, D54S, N55A, N97D

Group 3: I51L, T52G, G53D, D54S, N55A, N97R

Group 4: I96S, N97D, S98Q, G99H

Group 5: D95S, I96S, N97R, S98Q, G99H

Group 6: I51L, G53D

and/or the beta chain variable domain has at least one of the following groups of mutations

Group 1: N50W, Q51T, S53G

Group 2: S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G

Group 3: S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G, G100A

5 Group 4: S27K, Q28R, V29L, T30A, N50W, Q51T

Group 5: S27K, Q28R, V29L, T30A, N50W, Q51T S53G

Group 6: S27K, Q28R, V29L, T30A, N50W, Q51T S53G, G100A

Group 7: S27K, Q28R, V29L, T30A, M31L, N50W, Q51T

Group 8: T30A, N50W, G100A

10

Particular combinations of the groups of mutations may be as set out in the table below:

alpha chain		beta chain	
Group 1	I51L, T52G, G53D, D54S, N55A	Group 3	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G, G100A
Group 1	I51L, T52G, G53D, D54S, N55A	Group 7	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T
Group 3	I51L, T52G, G53D, D54S, N55A, N97R	Group 3	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T, S53G, G100A
Group 3	I51L, T52G, G53D, D54S, N55A, N97R	Group 7	S27K, Q28R, V29L, T30A, M31L, N50W, Q51T

(table 5)

15 Particular combinations of mutations may be present in the TCR of the invention as set out in the tables below:

Alpha Chain

Residues 27 - 32	Residues 50 - 57	Residues 91 - 107
VSGNPY	YITGDNLV	CAVRDSDQHAGSYQLTF
VSGNPY	YLGDSALV	CAVRDINSAGAGSYQLTF
VSGNPY	YITGDNLV	CAVRSSRQHAGSYQLTF
VSGNPY	YLGDSALV	CAVRDIDSGAGSYQLTF
VSGNPY	YLGDSALV	CAVRDIRSGAGSYQLTF
VSGNPY	YLTDDNLV	CAVRDINSAGAGSYQLTF

(table 6)

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Beta Chain

Residues 27 - 31	Residues 49 - 55	Residues 93 - 106
KRLAL	AWTGGEA	CSVGGSGGADTQYF
KRLAL	AWTGGEA	CSVGGSGAADTQYF
KRLAM	AWTGGEA	CSVGGSGGADTQYF
KRLAL	AWTGSEA	CSVGGSGGADTQYF
KRLAM	AWTGGEA	CSVGGSGAADTQYF
KRLAM	AWTGSEA	CSVGGSGGADTQYF
SQVTM	AWTGGEA	CSVGGSGGADTQYF
SQVAM	AWQGSEA	CSVGGSGAADTQYF

(table 7)

- 5 Particularly, the alpha and beta chain variable domains may comprise the following combinations of amino acid sequences:

Alpha chain			Beta Chain		
27-32	50-57	91-107	27-31	49-55	93-106
VSGNPY	YLGDSALV	CAVRDINSAGAGSYQLTF	KRLAL	AWTGGEA	CSVGGSGAADTQYF
VSGNPY	YLGDSALV	CAVRDINSAGAGSYQLTF	KRLAL	AWTGSEA	CSVGGSGGADTQYF
VSGNPY	YLGDSALV	CAVRDIRSGAGSYQLTF	KRLAL	AWTGGEA	CSVGGSGAADTQYF
VSGNPY	YLGDSALV	CAVRDIRSGAGSYQLTF	KRLAL	AWTGSEA	CSVGGSGGADTQYF

(table 8)

- 10 In certain embodiments, there are 1, 2, 3, 4, 5, 6, 7 or 8 mutations in alpha chain CDRs, for example 2 to 6 mutations, and/or 1, 2, 3, 4, 5, 6, 7, 8 or 9 mutations in the beta chain CDRs, for example 3 to 9 mutations. In some embodiments, the α chain variable domain of the TCR of the invention may comprise an amino acid sequence that has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98 % or at least 99% identity to the sequence of amino acid residues 1-117 of SEQ ID NO: 2, provided that the α
- 15 chain variable domain has at least one of the mutations outlined above. In some embodiments, the β chain variable domain of the TCR of the invention may comprise an amino acid sequence that has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98 % or at least 99% identity to the sequence of amino acid residues 1-115 of SEQ ID NO: 3, provided that the β chain variable domain has at least one of the mutations
- 20 outlined above. The alpha chain variable domain may comprise the amino acid sequence of any one of SEQ ID NO: 6-12 or 51. The beta chain variable domain may comprise the amino acid sequence of any one of SEQ ID NO: 13-23 or 52.

Mutations may also be made outside of the CDRs, such mutations may improve binding, but preferably improve purification yield and stability. Examples of such mutations in the alpha chain variable domain, with reference to the numbering of SEQ ID NO: 2 may be:

Residue no.	
Q10	L

(table 9)

5

and/or in the beta chain variable domain may be at least one of the following mutations with reference to the numbering of SEQ ID NO: 3:

Residue no.	
C13	K
L43	P

(table 10)

- 10 Mutations to a parental TCR may include those that are able to increase the binding affinity (K_D and/or binding half life) of the TCR to SLLMWITQC. Mutations may include those that are able to reduce the amount of non-specific binding, i.e. reduce binding to antigens in addition to binding to SLLMWITQC, or increase the specificity of the TCR binding to SLLMWITQC. Mutations may include those that increase efficiency of folding and/or manufacture. Some mutations may
- 15 contribute to each of these characteristics, others may contribute to affinity but not specificity, for example, or to specificity but not affinity etc.

- Within the scope of the invention are phenotypically silent variants of any TCR of the invention disclosed herein. As used herein the term "phenotypically silent variants" is understood to refer to a
- 20 TCR which incorporates one or more further amino acid changes in addition to those set out above which TCR has a similar phenotype to the corresponding TCR without said change(s). For the purposes of this application, TCR phenotype comprises antigen binding affinity (K_D and/or binding half-life) and antigen specificity. A phenotypically silent variant may have a K_D and/or binding half-life for the SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex within 20% of the measured K_D and/or
- 25 binding half-life of the corresponding TCR without said change(s), when measured under identical conditions (for example at 25°C and on the same SPR chip). Suitable conditions are further defined in Example 3. Antigen specificity is further defined below. As is known to those skilled in the art, it may be possible to produce TCRs that incorporate changes in the variable domains thereof compared to those detailed above without altering the affinity of the interaction with the
- 30 SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex. In particular, such silent mutations may be incorporated within parts of the sequence that are known not to be directly involved in antigen binding (e.g. outside the CDRs or parts of the CDRs that do not contact the peptide antigen). Such trivial variants are included in the scope of this invention. Those TCRs in which one or more conservative and/or tolerated substitutions have been made also form part of this invention.

Tolerated substitutions are also phenotypically silent but may not be conservative, as defined below. Tolerated substitutions may result in a decrease in affinity for the SLLMWITQC HLA-A*02 complex in comparison to a TCR not having a tolerated substitution. The decrease in affinity may be 5%, 10%, 15%, 20%, 25%, 30%, 40% or 50% of the TCR without the tolerated substitution. The
 5 decrease in affinity does not result in the affinity being less than (i.e. weaker than) 50µm.

The TCRs of the present invention may also include one or more conservative substitutions which have a similar amino acid sequence and/or which retain the same function. The skilled person is aware that various amino acids have similar properties and thus are 'conservative'. One or more
 10 such amino acids of a protein, polypeptide or peptide can often be substituted by one or more other such amino acids without eliminating a desired activity of that protein, polypeptide or peptide.

Thus the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is
 15 preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains);
 20 aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino
 25 acid substitutions. The present invention therefore extends to use of a TCR comprising an amino acid sequence described above but with one or more conservative substitutions in the sequence, such that the amino acid sequence of the TCR has at least 90% identity, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to the TCR comprising amino acids 1-117 of SEQ ID NOs: 2, 6-12 or 51 and/or amino acids 1-115 of SEQ ID NOs: 3, 13-23 or 52.

30

Particular combinations of the alpha chain variable domain and beta chain variable domain may be:

Alpha chain variable domain	Beta chain variable domain
SEQ ID NO: 7	SEQ ID NO: 15
SEQ ID NO: 7	SEQ ID NO: 18
SEQ ID NO: 12	SEQ ID NO: 15
SEQ ID NO: 12	SEQ ID NO: 18

(table 11)

Amino acid changes relative to the sequences given above can be made using any suitable technique e.g. by using site-directed mutagenesis or solid state synthesis.

It should be appreciated that amino acid substitutions within the scope of the present invention can be made using naturally occurring or non-naturally occurring amino acids. For example, it is contemplated herein that the methyl group on an alanine may be replaced with an ethyl group, and/or that minor changes may be made to the peptide backbone. Whether or not natural or synthetic amino acids are used, it is preferred that only L- amino acids are present.

"Identity" as known in the art is the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. While there exist a number of methods to measure identity between two polypeptide or two polynucleotide sequences, methods commonly employed to determine identity are codified in computer programs. Preferred computer programs to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, et al., Nucleic Acids Research, 12, 387 (1984), BLASTP, BLASTN, and FASTA (Atschul et al., J. Molec. Biol. 215, 403 (1990)).

One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment. A program like BLASTx will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of identity analysis are contemplated in the present invention.

The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the first sequence for best alignment with the sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences which results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (i.e., % identity = number of identical positions/total number of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci.

- USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. The NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to
- 5 nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules for use in the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilised as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between
- 10 molecules (Id.). When utilising BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another example of a mathematical algorithm utilised for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the CGC sequence alignment software package has
- 15 incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti (1994) Comput. Appl. Biosci., 10 :3-5; and FASTA described in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.
- 20 Mutations can be carried out using any appropriate method including, but not limited to, those based on polymerase chain reaction (PCR), restriction enzyme-based cloning, or ligation independent cloning (LIC) procedures. These methods are detailed in many of the standard molecular biology texts. For further details regarding polymerase chain reaction (PCR) and restriction enzyme-based cloning, see Sambrook & Russell, (2001) Molecular Cloning – A
- 25 Laboratory Manual (3rd Ed.) CSHL Press. Further information on ligation independent cloning (LIC) procedures can be found in Rashtchian, (1995) *Curr Opin Biotechnol* 6(1): 30-6.

The TCR of the second aspect may comprise an alpha chain framework 2 (FM2) region and an alpha chain framework 3 (FM3) region, wherein the FM2 and FM3 regions comprise SEQ ID NO:

30 47 and 48 respectively, and/or contain one or more conservative substitutions and/or up to three tolerated substitutions.

The TCR of the second aspect may comprise a beta chain FM2 region and a beta chain FM3 region, wherein the FM2 and FM3 regions comprise SEQ ID NOs:49 and 50 respectively, and/or

35 contain one or more conservative substitutions and/or up to three tolerated substitutions.

The TCR of the second aspect may comprise amino acids 1-117 of SEQ ID NO: 2 and/or amino acids 1-115 of SEQ ID NO: 3, which each may contain one or more conservative substitutions

and/or up to three tolerated mutations and/or one or more of the mutations defined in table 1 or table 2.

The TCRs of the invention have the property of binding the SLLMWITQC (SEQ ID NO: 1) HLA-A*02 complex. TCRs of the invention have been found to be highly specific for this epitope relative to other, irrelevant epitopes, and are thus particularly suitable as targeting vectors for delivery of therapeutic agents or detectable labels to cells and tissues displaying those epitopes. Specificity in the context of TCRs of the invention relates to their ability to recognise HLA-A*02 target cells that are positive for the peptide SLLMWITQC, whilst having minimal ability to recognise HLA-A*02 target cells that are negative for the peptide. To test specificity the TCRs may be in soluble form and/or may be expressed on the surface of T cells. Recognition may be determined by measuring the level of T cell activation in the presence of a TCR and target cells. In this case, minimal recognition of peptide negative target cells is defined as a level of T cell activation of less than 20% preferably less than 10%, and more preferably less than 5%, of the level produced in the presence of peptide positive target cells, when measured under the same conditions. For soluble TCRs of the invention, specificity may be determined at a therapeutically relevant TCR concentration. A therapeutically relevant concentration may be defined as a TCR concentration of 10^{-9} M or below, and/or a concentration of up to 100, or up to 1000, fold greater than the corresponding EC₅₀ value. Peptide positive cells may be obtained by peptide-pulsing or, more preferably, they may naturally present said peptide. Preferably, both peptide positive and peptide negative cells are human cells. Specificity can be measured, for example, in cellular assays such as those described in Examples 6-8. Specificity may additionally relate to the ability to bind to NY-ESO-1-HLA-A*02 complex and not multiple naturally-presented peptide HLA complexes, as determined by Biacore, for example. Preferably, binding to NY-ESO-1-HLA-A*02 complex is at least 400 fold greater than to other naturally-presented peptide HLA complexes.

TCRs of the invention may have a K_D for the NY-ESO-1-HLA-A*02 complex of greater than (i.e. stronger than) 50 μ M, for example between 50 μ M and 1 pM. Certain TCRs of the invention may have a K_D for the complex of from about 1 pM to about 50 nM, from about 1 pM to about 400 pM, from about 20 pM to about 200 pM. TCRs of the invention may have a binding half-life ($T_{1/2}$) for the complex in the range of from about 1 sec to about 60 h or greater, from about 30 min to about 60 h or greater, or from about 6 h to about 60 h or greater. TCRs that are for use as soluble therapeutics and/or diagnostics when coupled to a detectable label or therapeutic agent preferably have a K_D for the complex of from about 1 pM to about 200 pM, or from about 20 pM to about 100 pM, as determined using the BiAcCore method of Example 3, and/or a binding half-life for the complex of from about 2 h to 60 h or greater, or from about 20 h to about 60 h or greater, as determined using the BiAcCore method of Example 3. Certain TCRs of the invention may be suitable for adoptive therapy applications; such TCRs may have a K_D for the complex of from about 50 nM to about 50

μM, or from about 100 nM to about 1 μM and/or a binding half-life for the complex of from about 3 sec to about 12 min.

Certain TCRs of the invention have a binding affinity for, and/or a binding half-life for, the
5 SLLMWITQC -HLA-A*02 complex substantially higher than that of the native TCR.
Increasing the binding affinity of a native TCR often reduces the specificity of the TCR for its
peptide-MHC ligand, and this is demonstrated in Zhao Yangbing et al., The Journal of Immunology,
The American Association of Immunologists, US, vol. 179, No.9, 1 November 2007, 5845-5854.
However, the TCRs of the invention may remain specific for the SLLMWITQC -HLA-A*02 complex,
10 despite having substantially higher binding affinity than the native TCR.

Binding affinity (inversely proportional to the equilibrium constant K_D) and binding half-life
(expressed as $T_{1/2}$) can be determined using the Surface Plasmon Resonance (BIAcore) and /or
Octet method of Example 3 herein. It will be appreciated that doubling the affinity of a TCR results
15 in halving the K_D . $T_{1/2}$ is calculated as $\ln 2$ divided by the off-rate (k_{off}). Therefore, doubling of $T_{1/2}$
results in a halving in k_{off} . K_D and k_{off} values for TCRs are usually measured for soluble forms of
the TCR, i.e. those forms which are truncated to remove cytoplasmic and transmembrane domain
residues. Preferably the binding affinity or binding half-life of a given TCR is measured several
times, for example 3 or more times, using the same assay protocol and an average of the results is
20 taken.

For use as a targeting agent for delivering therapeutic agents to the antigen presenting cell the
TCR may be in soluble form (i.e. having no transmembrane or cytoplasmic domains). For stability,
TCRs of the invention, and preferably soluble αβ heterodimeric TCRs, may have an introduced
25 disulphide bond between residues of the respective constant domains, as described, for example,
in WO 03/020763. One or both of the constant domains present in an αβ heterodimer of the
invention may be truncated at the C terminus or C termini, for example by up to 15, or up to 10 or
up to 8 or fewer amino acids. The C terminus of the alpha chain extracellular constant region may
be truncated by 8 amino acids. For use in adoptive therapy, an αβ heterodimeric TCR may, for
30 example, be transfected as full length chains having both cytoplasmic and transmembrane
domains. TCRs for use in adoptive therapy may contain a disulphide bond corresponding to that
found in nature between the respective alpha and beta constant domains, additionally or
alternatively a non-native disulphide bond may be present.

The TCRs of the invention may be αβ heterodimers or may be in single chain format. Single chain
35 formats include αβ TCR polypeptides of the $V\alpha$ -L- $V\beta$, $V\beta$ -L- $V\alpha$, $V\alpha$ -C α -L- $V\beta$ or $V\alpha$ -L- $V\beta$ -C β types,
wherein $V\alpha$ and $V\beta$ are TCR α and β variable regions respectively, $C\alpha$ and $C\beta$ are TCR α and β
constant regions respectively, and L is a linker sequence. In certain embodiments single chain
TCRs of the invention may have an introduced disulphide bond between residues of the respective

constant domains, as described in WO 2004/033685. Single chain TCRs are further described in WO2004/033685; WO98/39482; WO01/62908; Weidanz et al. (1998) J Immunol Methods 221(1-2): 59-76; Hoo et al. (1992) Proc Natl Acad Sci U S A 89(10): 4759-4763; Schodin (1996) Mol Immunol 33(9): 819-829).

5

As will 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 binding characteristics of the TCR. All such trivial variants are encompassed by the present invention.

10

Alpha-beta heterodimeric TCRs of the invention usually comprise an alpha chain TRAC constant domain sequence and/or a beta chain TRBC1 or TRBC2 constant domain sequence. The alpha and beta chain constant domain sequences may be modified by truncation or substitution to delete the native disulphide bond between Cys4 of exon 2 of TRAC and Cys2 of exon 2 of TRBC1 or TRBC2. The alpha and/or beta chain constant domain sequence(s) may also be modified by

15

substitution of cysteine residues for Thr 48 of TRAC and Ser 57 of TRBC1 or TRBC2, the said cysteines forming a disulphide bond between the alpha and beta constant domains of the TCR.

In a further aspect, the present invention provides nucleic acid encoding a TCR of the first and/or second aspect of the invention. In some embodiments, the nucleic acid is cDNA. In some **embodiments, the invention provides nucleic acid comprising a sequence encoding an α chain variable domain of a TCR of the invention. In some embodiments, the invention provides nucleic acid comprising a sequence encoding a β chain variable domain of a TCR of the invention. The nucleic acid may be non-naturally occurring and/or purified and/or engineered.**

25

In another aspect, the invention provides a vector which comprises nucleic acid of the invention. Preferably the vector is a TCR expression vector.

The invention also provides a cell harbouring a vector of the invention, preferably a TCR expression vector. The vector may comprise nucleic acid of the invention encoding in a single open reading frame, or two distinct open reading frames, the alpha chain and the beta chain respectively. Another aspect provides a cell harbouring a first expression vector which comprises nucleic acid encoding the alpha chain of a TCR of the invention, and a second expression vector which comprises nucleic acid encoding the beta chain of a TCR of the invention. Such cells are particularly useful in adoptive therapy. The cells of the invention may be isolated and/or recombinant and/or non-naturally occurring and/or engineered.

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Since the TCRs of the invention have utility in adoptive therapy, the invention includes a non-naturally occurring and/or purified and/or or engineered cell, especially a T-cell, presenting a TCR

of the invention. The invention also provides an expanded population of T cells presenting a TCR of the invention. There are a number of methods suitable for the transfection of T cells with nucleic acid (such as DNA, cDNA or RNA) encoding the TCRs of the invention (see for example Robbins *et al.*, (2008) *J Immunol.* 180: 6116-6131). T cells expressing the TCRs of the invention will be
5 suitable for use in adoptive therapy-based treatment of cancer. As will be known to those skilled in the art, there are a number of suitable methods by which adoptive therapy can be carried out (see for example Rosenberg *et al.*, (2008) *Nat Rev Cancer* 8(4): 299-308).

Soluble TCRs of the invention are useful for delivering detectable labels or therapeutic agents to
10 antigen presenting cells and tissues containing antigen presenting cells. They may therefore be associated (covalently or otherwise) with a detectable label (for diagnostic purposes wherein the TCR is used to detect the presence of cells presenting the SLLMWITQC-HLA-A*02 complex); a therapeutic agent; or a PK modifying moiety (for example by PEGylation).

15 Detectable labels for diagnostic purposes include for instance, fluorescent labels, radiolabels, enzymes, nucleic acid probes and contrast reagents.

Therapeutic agents which may be associated with the TCRs of the invention include immunomodulators, radioactive compounds, enzymes (perforin for example) or chemotherapeutic
20 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 a TCR so that the compound is released slowly. This will prevent damaging effects during transport in the body and ensure that the toxin has maximum effect after binding of the TCR to the relevant antigen presenting cells.

25 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
30 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, temozolomide, topotecan, trimetreate glucuronate, auristatin E vincristine and doxorubicin;
- peptide cytotoxins, i.e. proteins or fragments thereof with the ability to kill mammalian cells. For example, ricin, diphtheria toxin, pseudomonas bacterial exotoxin A, DNase and RNase;
- radio-nuclides, i.e. unstable isotopes of elements which decay with the concurrent emission of one or more of α or β particles, or γ rays. For example, 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;

- immuno-stimulants, i.e. immune effector molecules which stimulate immune response. For example, cytokines such as IL-2 and IFN- γ ,
- Superantigens and mutants thereof;
- TCR-HLA fusions, e.g. fusion to a peptide-HLA complex, wherein said peptide is derived from a common human pathogen, such as Epstein Barr Virus (EBV);
- chemokines such as IL-8, platelet factor 4, melanoma growth stimulatory protein, etc;
- antibodies or fragments thereof, including anti-T cell or NK cell determinant antibodies (e.g. anti-CD3, anti-CD28 or anti-CD16);
- alternative protein scaffolds with antibody like binding characteristics
- complement activators;
- xenogeneic protein domains, allogeneic protein domains, viral/bacterial protein domains, viral/bacterial peptides.

One preferred embodiment is provided by a TCR anti-CD3 fusion comprising a TCR of the invention associated (usually by fusion to the N- or C-terminus of the alpha or beta chain) with an anti-CD3 antibody, or a functional fragment or variant of said anti-CD3 antibody. (Such TCR-anti-CD3 fusions may be termed ImmTACTM molecules) As used herein, the term TCR encompasses TCR-fusions, such as a TCR anti-CD3 fusion. As used herein, the term "antibody" encompasses such fragments and variants. Examples of anti-CD3 antibodies include but are not limited to OKT3, UCHT-1, BMA-031 and 12F6. Antibody fragments and variants/analogues which are suitable for use in the compositions and methods described herein include minibodies, Fab fragments, F(ab')₂ fragments, dsFv and scFv fragments, NanobodiesTM (these constructs, marketed by Ablynx (Belgium), comprise synthetic single immunoglobulin variable heavy domain derived from a camelid (e.g. camel or llama) antibody) and Domain Antibodies (Domantis (Belgium), comprising an affinity matured single immunoglobulin variable heavy domain or immunoglobulin variable light domain) or alternative protein scaffolds that exhibit antibody like binding characteristics such as Affibodies (Affibody (Sweden), comprising engineered protein A scaffold) or Anticalins (Pieris (Germany)), comprising engineered anticalins) to name but a few.

Linkage of the TCR and the anti-CD3 antibody may be direct, or indirect via a linker sequence. Linker sequences are usually flexible, in that they are made up primarily of amino acids such as glycine, alanine and serine which do not have bulky side chains likely to restrict flexibility. Usable or optimum lengths of linker sequences are easily determined. Often the linker sequence will be less than about 12, such as less than 10, or from 5-10 amino acids in length. Suitable linkers that may be used in TCR anti-CD3 fusions of the invention include, but are not limited to: GGGGS

(SEQ ID NO: 24), GGGSG (SEQ ID NO: 25), GGSGG (SEQ ID NO: 26), GSGGG (SEQ ID NO: 27), GSGGGP (SEQ ID NO: 28), GGEPS (SEQ ID NO: 29), GGEGGGP (SEQ ID NO: 30), and GGEGGGSEGGGS (SEQ ID NO: 31) (as described in WO2010/133828).

- 5 Specific embodiments of anti-CD3-TCR fusion constructs of the invention include those alpha and beta chain pairings shown in the table below.

	alpha chain SEQ ID NO:	beta chain SEQ ID NO:	Shown in
ImmTAC1	32	33	Figure 5
ImmTAC2	35	36	Figure 6
ImmTAC3	37	38	Figure 7
ImmTAC4	39	40	Figure 8

(table 7)

- 10 A particularly preferred anti-CD3-TCR fusion comprises alpha chain SEQ ID NO: 37 and beta chain SEQ ID NO: 38.

For some purposes, the TCRs of the invention may be aggregated into a complex comprising several TCRs to form a multivalent TCR complex. There are a number of human proteins that contain a multimerisation domain that may 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 be used for this kind of application. A multivalent TCR complex of the invention may have enhanced binding capability for the SLLMWITQC-HLA-A*02 complex compared to a non-multimeric wild-type or T cell receptor heterodimer of the invention. Thus, multivalent complexes of TCRs of the invention are also included within the invention. Such multivalent TCR complexes according to the invention are particularly useful for tracking or targeting cells presenting particular antigens *in vitro* or *in vivo*, and are also useful as intermediates for the production of further multivalent TCR complexes having such uses.

As is well-known in the art, TCRs may be subject to post translational modifications. Glycosylation is one such modification, which comprises the covalent attachment of oligosaccharide moieties to defined amino acids in the TCR chain. For example, asparagine residues, or serine/threonine residues are well-known locations for oligosaccharide attachment. The glycosylation status of a particular protein depends on a number of factors, including protein sequence, protein conformation and the availability of certain enzymes. Furthermore, glycosylation status (i.e. oligosaccharide type, covalent linkage and total number of attachments) can influence protein

function. Therefore, when producing recombinant proteins, controlling glycosylation is often desirable. Controlled glycosylation has been used to improve antibody based therapeutics. (Jefferis R., Nat Rev Drug Discov. 2009 Mar;8(3):226-34.). For soluble TCRs of the invention glycosylation may be controlled in vivo, by using particular cell lines for example, or in vitro, by chemical
5 modification. Such modifications are desirable, since glycosylation can improve pharmacokinetics, reduce immunogenicity and more closely mimic a native human protein (Sinclair AM and Elliott S., Pharm Sci. 2005 Aug; 94(8):1626-35).

For administration to patients, the TCRs or TCR anti-CD3 fusions of the invention (preferably
10 associated with a detectable label or therapeutic agent or expressed on a transfected T cell) or cells of the invention may be provided in a pharmaceutical composition together with one or more pharmaceutically acceptable carriers or excipients. Therapeutic or imaging TCRs, TCR fusions or cells, in accordance with the invention will usually be supplied as part of a sterile, pharmaceutical composition which will normally include a pharmaceutically acceptable carrier. This pharmaceutical
15 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.

20 The pharmaceutical composition may be adapted for administration by any appropriate route, such as parenteral (including subcutaneous, intramuscular, or intravenous), enteral (including oral or rectal), inhalation or intranasal routes. 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.

25 Dosages of the substances of the present invention 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. a suitable dose range for a soluble TCR of the invention associated with an anti-CD3 antibody may be between 25 ng/kg and 50 µg/kg. A physician will ultimately determine appropriate dosages to be
30 used.

TCRs, pharmaceutical compositions, vectors, nucleic acids and cells of the invention may be provided in substantially pure form, for example at least 80%, at least 85 %, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least
35 98%, at least 99% or 100% pure.

Also provided by the invention are:

- A TCR, TCR anti-CD3 fusion, nucleic acid or cell of the invention for use in medicine, preferably for use in a method of treating cancer.

- the use of a TCR, TCR anti-CD3 fusion, nucleic acid or cell of the invention in the manufacture of a medicament for treating cancer;
- a method of treating cancer in a patient, comprising administering to the patient a TCR, TCR anti-CD3 fusion, nucleic acid, or cell of the invention.

5

The cancer to be treated may be a synovial sarcoma, non small cell lung carcinoma (NSCLC), bladder tumour, gastric tumour, prostate tumour, colorectal tumour, breast tumour, ovarian tumour, oesophageal tumour, melanoma, multiple myeloma, hepatocellular carcinoma and head and neck tumour.

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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.

15 The invention is described below with reference to the following non-limiting figures and examples, in which:

Figure 1 provides amino acids sequences of the extracellular regions of native alpha and beta chain variable domains of the invention;

20

Figure 2 provides amino acid sequences of the soluble extracellular regions of native alpha and beta chains of the invention;

25 Figure 3 provides amino acid sequences of mutated TCR alpha chain variable regions of the invention;

Figure 4 provides amino acid sequences of mutated TCR beta chain variable regions of the invention;

30 Figure 5 provides amino acid sequences of ImmTAC molecules comprising TCR sequences of the invention;

Figure 6 provides amino acid sequences of ImmTAC molecules comprising TCR sequences of the invention;

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Figure 7 provides amino acid sequences of ImmTAC molecules comprising TCR sequences of the invention;

Figure 8 provides amino acid sequences of ImmTAC molecules comprising TCR sequences of the invention;

Figure 9 shows potent and specific binding of ImmTAC molecules of the invention;

5

Figure 10 shows further potency testing for 2 ImmTAC molecules of the invention;

Figure 11 shows further specificity testing for 3 ImmTAC molecules of the invention; and

10 Figure 12 shows tumour cell killing by ImmTAC redirected T cells.

Examples

15 **Example 1 – Expression, refolding and purification of soluble TCRs**

DNA sequences encoding the alpha and beta extracellular regions of soluble TCRs of the invention were cloned separately into pGMT7-based expression plasmids using standard methods (as described in Sambrook, et al. *Molecular cloning*. Vol. 2. (1989) New York: Cold spring harbor laboratory press). The expression plasmids were transformed separately into *E. coli* strain Rosetta
20 (BL21pLysS), and single ampicillin-resistant colonies were grown at 37°C in TYP (+ ampicillin 100 µg/ml) medium to an OD₆₀₀ of ~0.6-0.8 before inducing protein expression with 0.5 mM IPTG. Cells were harvested three hours post-induction by centrifugation. Cell pellets were lysed with BugBuster protein extraction reagent (Merck Millipore) according to the manufacturer's instructions. Inclusion body pellets were recovered by centrifugation. Pellets were washed twice in Triton buffer
25 (50 mM Tris-HCl pH 8.1, 0.5% Triton-X100, 100 mM NaCl, 10 mM NaEDTA) and finally resuspended in detergent free buffer (50 mM Tris-HCl pH 8.1, 100 mM NaCl, 10 mM NaEDTA). Inclusion body protein yield was quantified by solubilising with 6 M guanidine-HCl and measuring OD₂₈₀. Protein concentration was then calculated using the extinction coefficient. Inclusion body purity was measured by solubilising with 8M Urea and loading ~2µg onto 4-20% SDS-PAGE under
30 reducing conditions. Purity was then estimated or calculated using densitometry software (Chemidoc, Biorad). Inclusion bodies were stored at +4°C for short term storage and at -20°C or -70°C for longer term storage.

For soluble TCR refolding, α and β chain-containing inclusion bodies were first mixed and diluted into 10 ml solubilisation/denaturation buffer (6 M Guanidine-hydrochloride, 50 mM Tris HCl pH 8.1,
35 100 mM NaCl, 10 mM EDTA, 20 mM DTT) followed by incubation for 30 min at 37°C. Refolding was then initiated by further dilution into 1 L of refold buffer (100 mM Tris pH 8.1, 400 mM L-Arginine HCL, 2 mM EDTA, 4 M Urea, 10 mM cysteamine hydrochloride and 2.5 mM cystamine dihydrochloride) and the solution mixed well. The refolded mixture was dialysed against 10 L H₂O

for 18-20 hours at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$. After this time, the dialysis buffer was twice replaced with 10 mM Tris pH 8.1 (10 L) and dialysis continued for another 15 hours. The refold mixture was then filtered through 0.45 μm cellulose filters.

Purification of soluble TCRs was initiated by applying the dialysed refold onto a POROS® 50HQ anion exchange column and eluting bound protein with a gradient of 0-500mM NaCl in 20 mM Tris pH 8.1 over 50 column volumes using an Akta® purifier (GE Healthcare). Peak TCR fractions were identified by SDS PAGE before being pooled and concentrated. The concentrated sample was then applied to a Superdex® 75HR gel filtration column (GE Healthcare) pre-equilibrated in Dulbecco's PBS buffer. The peak TCR fractions were pooled and concentrated and the final yield of purified material calculated.

Example 2 – Expression, refolding and purification of ImmTAC molecules (soluble TCR-anti CD3 fusion proteins)

ImmTAC preparation was carried out as described in Example 1, except that the TCR beta chain was fused via a linker to an anti-CD3 single chain antibody. In addition a cation exchange step was performed during purification following the anion exchange. In this case the peak fractions from anion exchange were diluted 20 fold in 20mM MES (pH6.5), and applied to a POROS® 50HS cation exchange column. Bound protein was eluted with a gradient of 0-500 mM NaCl in 20mM MES. Peak ImmTAC fractions were pooled and adjusted to 50mM Tris pH 8.1, before being concentrated and applied directly to the gel filtration matrix as described in Example 1.

Example 3 – Binding characterisation

Binding analysis of purified soluble TCRs and ImmTAC molecules to the relevant peptide-HLA complex was carried out by surface plasmon resonance, using a BIAcore 3000 or BIAcore T200 instrument, or by biolayer interferometry, using a ForteBio Octet instrument). Biotinylated class I HLA-A*02 molecules were refolded with the peptide of interest and purified using methods known to those in the art (O'Callaghan et al. (1999). Anal Biochem 266(1): 9-15; Garboczi, et al. (1992). Proc Natl Acad Sci USA 89(8): 3429-3433). All measurements were performed at 25°C in Dulbecco's PBS buffer, supplemented with 0.005% P20.

30 *BIAcore*

Biotinylated peptide-HLA monomers were immobilized on to streptavidin-coupled CM-5 sensor chips. Equilibrium binding constants were determined using serial dilutions of soluble TCR / ImmTAC injected at a constant flow rate of $30 \mu\text{l min}^{-1}$ over a flow cell coated with ~200 response

units (RU) of peptide-HLA-A*02 complex. Equilibrium responses were normalised for each TCR concentration by subtracting the bulk buffer response on a control flow cell containing an irrelevant peptide-HLA. The K_D value was obtained by non-linear curve fitting using Prism software and the Langmuir binding isotherm, $\text{bound} = C \cdot \text{Max} / (C + K_D)$, where "bound" is the equilibrium binding in RU at injected TCR concentration C and Max is the maximum binding.

For high affinity interactions, binding parameters were determined by single cycle kinetics analysis. Five different concentrations of soluble TCR / ImmTAC were injected over a flow cell coated with ~100 - 200 RU of peptide-HLA complex using a flow rate of 50-60 $\mu\text{l min}^{-1}$. Typically, 60-120 μl of soluble TCR / ImmTAC was injected at a top concentration of 100-200 nM, with successive 2 fold dilutions used for the other four injections. The lowest concentration was injected first. To measure the dissociation phase buffer was then injected until $\geq 10\%$ dissociation occurred, typically after 1 - 3 hours. Kinetic parameters were calculated using BIAevaluation® software. The dissociation phase was fitted to a single exponential decay equation enabling calculation of half-life. The equilibrium constant K_D was calculated from $k_{\text{off}}/k_{\text{on}}$.

15 *Octet*

Biotinylated peptide-HLA monomers were captured to 1 nm on to (SA) streptavidin biosensors (Pall ForteBio) pre-immobilised with streptavidin. The sensors were blocked with free biotin (2 μM) for 2 minutes. Equilibrium binding constants were determined by immersing the loaded biosensors into soluble TCR / ImmTAC serially diluted in a 96-well or 384-well sample plate. Plate shaking was set to 1000 rpm. For low affinity interactions (μM range) a short association (~2 minutes) and a short dissociation time (~2 minutes) was used. Binding curves were processed by double reference subtraction of reference biosensors loaded with irrelevant pHLA using Octet Data Analysis Software (Pall ForteBio). Responses (nm) at equilibrium were used to estimate the K_D value from steady state plots fitted to the equation $\text{Response} = R_{\text{max}} \cdot \text{conc} / (K_D + \text{conc})$, where "response" is the equilibrium binding in nm at each TCR concentration (conc) and R_{max} is the maximum binding response at pHLA saturation.

For high affinity interactions (nM - pM range), kinetic parameters were determined from binding curves at ≥ 3 TCR / ImmTAC concentrations typically 10 nM, 5 nM and 2.5 nM. The association time was 30 minutes and the dissociation time 1 – 2 hours. Binding curves were processed by double reference subtraction of reference biosensors loaded with irrelevant pHLA and blocked with biotin. Kinetic parameters k_{on} and k_{off} were calculated by global fitting directly to the binding curves using Octet Data Analysis Software (Pall ForteBio). K_D was calculated from $k_{\text{off}}/k_{\text{on}}$ and the dissociation half-life was calculated from $t_{1/2} = 0.693/k_{\text{off}}$.

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Example 4 – Binding characterisation of a soluble non-mutated TCR of the invention

A soluble wild-type TCR was prepared according to the methods described in Example 1 and binding to pHLA analysed according to Example 3. The amino acid sequences of the alpha and beta chains corresponded to those shown in Figure 2. Soluble biotinylated HLA-A*02 was prepared with either the wild-type (SLLMWITQC) or heteroclitic NY-ESO-1 peptide (SLLMWITQV SEQ ID NO: 34), and immobilised onto a Biacore sensor chip. K_D values were determined to be 5.2 μ M and 4.3 μ M respectively. No significant binding was detected against 15 irrelevant peptide HLA-A*02 complexes.

These data indicate that the TCR binds to the target with a suitable affinity and specificity. These TCR chains therefore provide a useful scaffold for the identification of further TCRs of the invention.

Example 5 – Binding characterisation soluble high affinity TCRs and ImmTAC molecules of the invention

Soluble mutated TCRs and ImmTAC molecules were prepared as described in Examples 1 and 2, and binding characteristics determined according to Example 3. The TCR alpha and or beta chains contained mutations in at least one CDR region relative to the CDR sequences shown in Figure 2 (SEQ ID NOs:41 to 46). The amino acid sequences of certain mutated TCR alpha and beta chain variable regions of the invention are provided in Figures 4 and 5 respectively. The table below provides binding characteristics for soluble TCRs and/or ImmTAC molecules comprising the indicated alpha and beta variable regions. Binding was measured using the heteroclitic NY-ESO-1 peptide (SLLMWITQV).

Alpha chain SEQ ID NO:	Beta chain SEQ ID NO:	Format (soluble / ImmTAC)	Method (Biacore / Octet)	Binding parameters	
				KD	T _{1/2}
(a12) 6	(b12) 14	ImmTAC	Biacore	202 pM	19.2 h
(a12) 6	(b5) 13	soluble	Biacore	1.5 nM	163.6 min
(a12) 6	(b52) 15	ImmTAC	Biacore	244 pM	14.2 h
2	(b5) 13	soluble	Octet	nd	1.3 min
2	(b12) 14	soluble	Biacore	3.8 nM	72.33 min
2	(b12) 14	ImmTAC	Biacore	1.6 nM	1.92 h
(a24I) 8	(b52) 15	ImmTAC	Biacore	65 pM	33.54 h
(a24I) 8	(b65I) 19	ImmTAC	Octet	324 pM	8.71 h
(a24I) 8	(b12) 14	ImmTAC	Biacore	125 pM	17.9 h
(a24I) 8	(b65) 18	ImmTAC	Octet	325 pM	6.8 h

(a24l) 8	(b56) 16	ImmTAC	Octet	253 pM	6.65 h
(a24l) 8	(b56l) 17	ImmTAC	Octet	249 pM	9.05 h
(a24l) 8	(b67) 20	ImmTAC	Octet	252 pM	9.49 h
(a24l) 8	(b67l) 21	ImmTAC	Octet	256 pM	10.08 h
(a24l) 8	(b68) 22	ImmTAC	Octet	273 pM	7.38 h
(a24l) 8	(b68l) 23	ImmTAC	Octet	303 pM	6.59 h
(a24l) 8	(b5) 13	soluble	Biacore	2.7 nM	54.8 min
(a24) 7	(b52) 15	ImmTAC ¹	Biacore	18.6 pM	78 h
(a24) 7	(b65) 18	ImmTAC ²	Biacore	76 pM	24.2 h
(a28) 9	(b12) 14	ImmTAC	Biacore	120 pM	22.8 h
(a28) 9	(b5) 13	soluble	Biacore	1.7 nM	155.8 min
(a28) 9	(b52) 15	ImmTAC	Octet	361 pM	7.13 h
(a78l) 10	(b52) 15	ImmTAC	Octet	210 pM	7.6 h
(a78l) 10	(b67) 20	ImmTAC	Octet	173 pM	6.9 h
(a78l) 10	(b68) 22	ImmTAC	Octet	334 pM	4.7 h
(a78l) 10	(b12) 14	ImmTAC	Octet	272 pM	7.3 h
(a82l) 11	(b65) 18	ImmTAC	Biacore	135 pM	17.7 h
(a82l) 11	(b52) 15	ImmTAC	Biacore	30.8 pM	72 h
(a82l) 11	(b67) 20	ImmTAC	Octet	226 pM	8.5 h
(a82l) 11	(b68) 22	ImmTAC	Octet	476 pM	4.1 h
(a82l) 11	(b12) 14	ImmTAC	Octet	547 pM	5.3 h
(a82) 12	(b52) 15	ImmTAC ³	Biacore	40 pM	64.2 h
(a82) 12	(b65) 18	ImmTAC ⁴	Biacore	166 pM	18.7 h
(a86) 51	(b71) 52	ImmTAC	Biacore	39.8	15.7 h

nd = not determined

¹ Corresponds to ImmTAC1 from Example 6, full alpha and beta chain sequences are provided in Figure 5

² Corresponds to ImmTAC2 from Example 6, full alpha and beta chain sequences are provided in Figure 6

³ Corresponds to ImmTAC3 from Example 6, full alpha and beta chain sequences are provided in Figure 7

5 ⁴ Corresponds to ImmTAC4 from Example 6, full alpha and beta chain sequences are provided in Figure 8

These data demonstrate TCR alpha and beta chain sequences of the invention produce soluble TCRs and ImmTAC molecules with binding characteristics suitable for the development of immunotherapeutic reagents.

10

Example 6 – Potent and specific T cell redirection by ImmTAC molecules of the invention

ImmTAC molecules containing alpha and beta variable chain sequences of the invention were tested for their ability to mediate potent and specific redirection of CD3+ T cells by ELISPOT assay, using interferon- γ (IFN- γ) secretion as a read out for T cell activation.

- 5 The sequences of the alpha and beta chains of the four ImmTAC molecules tested are provided in Figures 5-8.

Assays were performed using a human IFN- γ ELISPOT kit (BD Biosciences). Target cells were prepared at a density of 1×10^6 / ml in assay medium (RPMI 1640 containing 10% heat inactivated FBS and 1% penicillin-streptomycin-L-glutamine) and plated at 50,000 cells per well in a volume of 10 50 μ l. The following target cell lines were used in this example:

- NCI-H1755 (NY-ESO-1^{+ve}; HLA-A*02^{+ve}) human lung cancer cell line (supplied by ATCC, cat. no: CRL-5892)
- HAo5 (NY-ESO-1^{-ve}; HLA-A*02^{+ve}) human cardiac cells (supplied by Promocell, cat. no: C-12271)

15

Peripheral blood mononuclear cells (PBMC), isolated from fresh donor blood, were used as effector cells and plated at a concentration of 40,000 cells per well in a volume of 50 μ l. Varying concentrations of ImmTAC were used, spanning the anticipated clinically relevant range, and added to the well in a volume of 50 μ l.

20

Plates were prepared according to the manufacturer's instruction. Target cells, effector cells and ImmTAC molecules were added to the relevant wells and made up to a final volume of 200 μ l with assay medium. All reactions were performed in triplicate. Control wells were also prepared with the omission of either ImmTAC, effector cells, or target cells. The plates were then incubated overnight 25 (37°C / 5%CO₂). The next day the plates were washed three times with wash buffer (1xPBS sachet, containing 0.05% P20, made up in deionised water). Primary detection antibody was then added to each well in a volume of 50 μ l. Plates were incubated at room temperature for 2 hours prior to being washed again three times. Secondary detection was performed by adding 50 μ l of diluted streptavidin-HRP to each well and incubating at room temperature for 1 hour and the 30 washing step repeated. Plates were then washed twice with 200 μ l PBS (pH 7.4). No more than 15 min prior to use, one drop (20 μ l) of AEC chromogen was added to each 1 ml of AEC substrate and mixed and 50 μ l added to each well. Spot development was monitored regularly and plates were washed in tap water to terminate the development reaction. The plates were then allowed to dry at room temperature for at least 2 hours prior to counting the spots using an CTL analyser with 35 Immunospot software (Cellular Technology Limited).

The data presented in Figure 9 show that ImmTAC molecules containing alpha and beta variable chain sequences of the invention are able to mediate potent T cell redirection against HLA-A*02^{+ve} cancer cells expressing target antigen. No activity was observed against HLA-A*02^{+ve} antigen negative cells. These data indicate that ImmTAC molecules are target cell specific within the clinically relevant concentration range (≤ 1 nM).

Further assessment of potency was carried out for ImmTAC molecules 3 and 4 to determine EC50 values. ELISpot assays were performed as described above with ImmTAC concentrations ranging from 10^{-13} M to 10^{-8} M. Data were analysed using Prism 5.0 software (GraphPad) to calculate EC50 values. Values were determined as 95 pM and 121 pM for ImmTAC molecules 3 and 4 respectively (Figure 10). These data confirm the ability of these ImmTAC molecules to mediate a potent redirected T cell response.

Example 7 - Further specificity testing of ImmTAC molecules of the invention

Further specificity testing of ImmTAC molecules was carried out against a panel of normal cells. ImmTAC molecules 2-4 (Figures 6-8) are used in this example. Interferon- γ (IFN- γ) secretion was used as a read out for T cell activation.

Assays were performed using an IFN- γ DuoSet ELISA kit (R&D Systems, Cat No: DY285) and carried out as instructed by the manufacturer. Briefly, IFN- γ was diluted to 10,000 pg/ml and 2 fold dilutions made to produce a standard curve. Target cells were counted and plated at 10,000 cells per well in 10 ul in assay media. ImmTAC molecules were diluted to give final concentrations of 2 nM and 1 nM in 10 ul per well. A control sample without ImmTAC was also prepared. Effector PBMCs were thawed and plated at 10,000 cells/well in 10 ul. The plates were incubated for 48 h before being developed and read.

In this example, NCI-H1755 cells were used as an antigen positive control and HTC-116 cells were used as an antigen negative control. The cell panel included cardiomyocytes (CM12, CM5 and CM10), aortic endothelial cells (HAo5), airway epithelial cells (HCAEC2 and HCAEC5) skeletal muscle myoblasts (HSkMM3) and HPF9 cells. All cell lines were HLA-A*02^{+ve}. Assays were performed in triplicate.

The data presented in Figure 11 demonstrate minimal IFN- γ production in the presence of cell lines derived from normal tissues, relative to antigen positive cancer cell lines, within the therapeutically relevant ImmTAC concentration range. These data indicate that ImmTAC molecules of the invention have a high level of specificity and are therefore particularly suitable for therapeutic use.

Example 8 - Potent killing of tumour cells by ImmTAC redirected T cells

The ability of ImmTAC molecules of the invention to mediate potent redirected T cell killing of antigen positive tumour cells was investigated using the IncuCyte platform (Essen BioScience).

- 5 This assay allows real time detection by microscopy of the release of Caspase-3/7, a marker for apoptosis.

Assays were performed using the CellPlayer 96-well Caspase-3/7 apoptosis assay kit (Essen BioScience, Cat. No.4440) and carried out according the manufacturers protocol. Briefly, target
10 cells (NCI-H1755 - NYESO^{+ve} HLA A*02^{+ve} or HCT-116 - NYESO^{-ve} HLA A*02^{+ve}) were plated at 5000 cells per well and incubated overnight to allow them to adhere. ImmTAC solutions were prepared at concentrations between 2.16 nM and 8.8 pM. 25 ul of each concentration was added to the relevant well. PBMCs were used as effector cells and plated at 50,000 per well in 50 ul. A control sample without ImmTAC was also prepared. NucView assay reagent was made up at 30
15 uM and 25 ul added to every well (giving 5 uM final conc). The plate was placed in the IncuCyte instrument and images taken every 2 hours (1 image per well) over 3 days. The number of apoptotic cells in each image was determined and recorded as no. objects per mm². Assays were performed in triplicate.

- 20 The data presented in Figure 12 show real-time killing of tumour cells by ImmTAC redirected T cells. Results are presented for ImmTAC3 and ImmTAC4. Both ImmTAC molecules shows T cell redirected killing of antigen positive tumour cells at concentrations as low as 26 pM . ImmTAC3 shows T cell redirected killing below 10 pM. Low level killing of antigen negative cells is only observed at the highest concentration (2.16 nM).

25

These data confirm that ImmTAC3 and immTAC4 mediate potent redirected T cell killing of antigen positive tumour cells within the therapeutically relevant concentration range.