

AFFINITY ENTITIES COMPRISING A TCR-LIKE ANTIBODY BINDING DOMAIN WITH HIGH AFFINITY AND FINE SPECIFICITY AND USES OF SAME

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to affinity entities comprising a TCR-like antibody binding domain with high affinity and fine specificity and uses of same.

Tumor and virus-infected cells are recognised by CD8⁺ cytotoxic T cells that, in response, are activated to eliminate these cells. In order to be activated, the clonotypic T-cell receptor (TCR) needs to encounter a specific peptide antigen presented by the membrane surface major histocompatibility complex (MHC) molecule. Cells that have undergone malignant transformation or viral infection present peptides derived from tumour-associated antigens or viral proteins on their MHC class I molecules. Therefore, disease-specific MHC-peptide complexes are desirable targets for immunotherapeutic approaches. One such approach transforms the unique fine specificity but low intrinsic affinity of TCRs to MHC-peptide complexes into high-affinity soluble antibody molecules endowed with a TCR-like specificity towards tumour or viral epitopes. These antibodies, termed TCR-like antibodies, are being developed as a new class of immunotherapeutics that can target tumour and virus-infected cells and mediate their specific killing. In addition to their therapeutic capabilities, TCR-like antibodies are being developed as diagnostic reagents for cancer and infectious diseases, and serve as valuable research tools for studying MHC class I antigen presentation.

25 SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 Heavy		
Chain (HC)	SEQ ID NO: 309	SYGVH
CDR2 HC	SEQ ID NO: 310	VIWAGGTTNYSALMS
CDR3 HC	SEQ ID NO: 311	DGHFHFDF
the CDR1 Light		
Chain (LC)	SEQ ID NO: 303	RASDIIYSNLA
CDR2 LC	SEQ ID NO: 304	AATNLAA

CDR3 LC SEQ ID NO: 305 QHFWGSSIS
the affinity binding entity capable of binding HLA-A2/Tyr_{D369-377} in an MHC restricted manner.

According to an aspect of some embodiments of the present invention there is provided an affinity binding entity comprising an antigen binding domain comprising

5 CDR sequences which are N-C ordered:

CDR1 Heavy Chain (HC)	SEQ ID NO: 293	TSGMGVS
CDR2 HC	SEQ ID NO: 294	HIYWDDDKRYNPSLKS
CDR3 HC	SEQ ID NO: 295	KDYGSSFYAMHY
the the CDR1 Light Chain (LC)	SEQ ID NO: 287	KASQDIHNYIA
CDR2 LC	SEQ ID NO: 288	YTSTLQP
CDR3 LC	SEQ ID NO: 289	LQYDNLWT

the affinity binding entity capable of binding HLA-A2/Tyr_{D369-377} in an MHC restricted manner.

According to an aspect of some embodiments of the present invention there is provided an affinity binding entity comprising an antigen binding domain comprising

10 CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 325	SYDMS
CDR2 HC	SEQ ID NO: 326	YMSSGGGTYPDTVKG
CDR3 HC	SEQ ID NO: 327	HDEITNFDY
CDR1 LC	SEQ ID NO: 319	RASQSI NSLH
CDR2 LC	SEQ ID NO: 320	YASQSI
CDR3 LC	SEQ ID NO: 321	QQSYSWPLT

the affinity binding entity capable of binding HLA-A2/ WT₁₂₆₋₁₃₄ in an MHC restricted manner.

According to an aspect of some embodiments of the present invention there is provided an affinity binding entity comprising an antigen binding domain comprising

15 CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 341	GYWIE
CDR2 HC	SEQ ID NO: 342	EILPGSGGTNYNEKFKG
CDR3 HC	SEQ ID NO: 343	DSNSFTY
CDR1 LC	SEQ ID NO: 335	SVSSVDYIH
CDR2 LC	SEQ ID NO: 336	STSILAS
CDR3 LC	SEQ ID NO: 337	QQRSSYT

the affinity binding entity capable of binding HLA-A2/ MAGE-A₄₃₂₈₋₃₄₃ in an MHC restricted manner.

According to an aspect of some embodiments of the present invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 357	FSSSWMN
CDR2 HC	SEQ ID NO: 358	RIYPGDGDTNYNEKFKG
CDR3 HC	SEQ ID NO: 359	EATTVVAPYYFDY
CDR1 LC	SEQ ID NO: 351	RASENIYRNLA
CDR2 LC	SEQ ID NO: 352	AATNLAD
CDR3 LC	SEQ ID NO: 353	QHFWGTPLT

the affinity binding entity capable of binding HLA-A2/ MAGE-A9₃₄₄₋₃₅₉ in an MHC restricted manner.

According to an aspect of some embodiments of the present invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 373	DYNMD
CDR2 HC	SEQ ID NO: 374	DINPNYDTTTYNQKFKG
CDR3 HC	SEQ ID NO: 375	RNYGNYVGDF
CDR1 LC	SEQ ID NO: 367	KASQRVNNDVA
CDR2 LC	SEQ ID NO: 368	YASNRYT
CDR3 LC	SEQ ID NO: 369	QQDYSSPFT

the affinity binding entity capable of binding HLA-A2/ PAP₃₆₀₋₃₇₅ in an MHC restricted manner.

According to some embodiments of the invention, the affinity binding entity is selected from the group consisting of an antibody, a CAR and a TCR.

According to some embodiments of the invention, the affinity binding entity is an antibody.

According to some embodiments of the invention, the affinity binding entity is a TCR.

According to some embodiments of the invention, the affinity binding entity is a CAR.

According to some embodiments of the invention, the affinity binding entity is a soluble entity.

According to some embodiments of the invention, the affinity binding entity is a humanized antibody.

According to some embodiments of the invention, the affinity binding entity comprises a therapeutic moiety.

According to some embodiments of the invention, the affinity binding entity comprises a detectable moiety.

5 According to some embodiments of the invention, the antibody is a single chain antibody, a bi-specific antibody or a full length antibody.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding the affinity binding entity.

10 According to an aspect of some embodiments of the present invention there is provided an expression vector comprising the polynucleotide operally linked to a cis-acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided a cell comprising the polynucleotide or the expression vector.

15 According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the affinity binding entity, the vector or the cell.

According to an aspect of some embodiments of the present invention there is provided a method of detecting a cancer cell, comprising contacting the cell with the antibody, under conditions which allow immunocomplex formation, wherein a presence of the immunocomplex or level thereof is indicative of the cancer cell.

20 According to an aspect of some embodiments of the present invention there is provided a method of diagnosing and treating cancer in a subject in need thereof, comprising:

- 25 (a) detecting the presence of cancer cells in the subject according to the method;
- (b) diagnosing the subject as having cancer when cancer cells are detected;
- (c) treating the subject with an anti-cancer therapy.

According to an aspect of some embodiments of the present invention there is provided a method of diagnosing cancer in a subject in need thereof, comprising contacting a cell of the subject with the antibody, under conditions which allow

immunocomplex formation, wherein a presence of the immunocomplex or level thereof is indicative of the cancer.

According to some embodiments of the invention, the cell is a skin cell.

According to an aspect of some embodiments of the present invention there is provided a method of treating a cancer, comprising administering to a subject in need thereof a therapeutically effective amount of the affinity binding entity, the vector or the cell, thereby treating the cancer.

According to an aspect of some embodiments of the present invention there is provided use of the affinity binding entity, the vector or the cell in the manufacture of a medicament for treating cancer.

According to some embodiments of the invention, the affinity binding entity is for TyrD the cancer is selected from the group consisting of melanoma and glioblastoma.

According to some embodiments of the invention, the affinity binding entity is for WT1 the cancer is selected from the group consisting of chronic myelocytic leukemia, multiple myeloma (MM), acute lymphoblastic leukemia (ALL), acute myeloid/myelogenous leukemia (AML), myelodysplastic syndrome (MDS), mesothelioma, ovarian cancer, gastrointestinal cancers e.g., colorectal cancer adenocarcinoma, thyroid cancer, breast cancer, lung cancer (e.g., non small cell lung cancer), melanoma, osteosarcoma, endometrial cancer, prostate cancer and glioblastoma.

According to some embodiments of the invention, when the affinity binding entity is for MAGE-A4 the cancer is selected from the group consisting of melanoma, ovarian cancer, T cell leukemia/lymphoma (e.g., ATLL), testicular cancer, head and neck cancer, bladder cancer and esophagus cancer.

According to some embodiments of the invention, the affinity binding entity is for MAGE-A9 the cancer is selected from the group consisting of renal cell carcinoma, bladder cancer, breast cancer and hepatocellular carcinoma.

According to some embodiments of the invention, the affinity binding entity is for PAP the cancer is selected from the group consisting of prostate cancer.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which

the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

Figure 1: Apparent binding affinity determination of TCR-like antibodies targeting HLA-A2/Tyrosinase complexes. Purified IgGs were immobilized indirectly to the SPR sensor chip with anti-mouse or human IgG. Analyte was purified recombinant single-chain HLA-A2/Tyrosinase complexes generated by in vitro refolding of E.coli expressed scHLA-A2 complexes.

Figure 2: Epitope specificity determination of TCR-like antibodies by Alanine scanning. The Tyrosinase peptide sequence was substituted with Alanine at positions 1,2,3,4,5,6,7, and 8. The Ala mutated peptides were synthesized and loaded onto T2 cells APCs at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding of TCR-like antibodies at a concentration of 10µg/ml was accessed by flow cytometry and binding intensity as measured by mean fluorescence intensity was measured and compared with the binding intensity to WT native Tyrosinase peptide. The relative effect of each position Ala substitution was evaluated as percentage to the binding to WT peptide.

Figure 3: Binding of D11 and D7 TCR-like antibodies to T2 APCs loaded with tyrosinase peptide and control HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-

mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 4: Binding of D11 and D7 TCR-like antibodies to T2 APCs loaded with tyrosinase peptide and control HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 5: Binding of D11 TCR-like antibody to T2 APCs loaded with tyrosinase peptide and control HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 6: Binding of D7 TCR-like antibody to T2 APCs loaded with tyrosinase peptide and control HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 7: Binding of MC1 TCR-like antibody to T2 APCs loaded with tyrosinase peptide and control HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 8: Binding of MC1 TCR-like antibody to melanoma cells that express HLA-A2 and Tyrosinase. Melanoma cells were monitored by flow cytometry for binding of TCR-like antibody MC1 using secondary PE-labeled anti-human IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 9: Binding of MC1 TCR-like antibody to HLA-A2+ and Tyrosinase antigen positive or negative cells. Tumor cells that express HLA-A2 and are positive or negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody MC1 using secondary PE-labeled anti-human IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 10: Binding of D11 and D7 TCR-like antibodies to HLA-A2+ and Tyrosinase antigen positive or negative cells. Tumor cells that express HLA-A2 and are positive or negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody MC1 using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 11: Binding of D11 and D7 TCR-like antibodies to HLA-A2+ and Tyrosinase negative cells. Tumor cells that express HLA-A2 and are negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody MC1 using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 12: Comparative Binding of D11, D7, and MC1 TCR-like antibodies to HLA-A2+ and Tyrosinase positive or negative cells. Tumor cells that express HLA-A2 and are positive or negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody D11, D7, and MC1 using secondary PE-labeled anti-mouse IgG.

Figure 13: Binding of D11 TCR-like antibody to HLA-A2+ / Tyrosinase negative normal primary cells. Primary normal cells of histological origin as indicated that express HLA-A2 and are negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody D11, using secondary PE-labeled anti-mouse IgG. MAb BB7.2 was used to monitor expression of HLA-A2.

Figure 14: Binding of D11 TCR-like antibody to HLA-A2+ / Tyrosinase negative normal primary cells. Primary normal cells of histological origin as indicated that express HLA-A2 and are negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody D11, using secondary PE-labeled anti-mouse IgG.

Figure 15: Binding of D7 TCR-like antibody to HLA-A2+ / Tyrosinase negative normal primary cells. Primary normal cells of histological origin as indicated that

express HLA-A2 and are negative for Tyrosinase were monitored by flow cytometry for binding of TCR-like antibody D7, using secondary PE-labeled anti-mouse IgG.

Figure 16: Binding of BB7.2 to normal primary cells. Primary normal cells of histological origin were monitored by flow cytometry for expression of HLA-A2 using MAb BB7.2 and secondary PE-labeled anti-mouse IgG.

Figure 17: Binding of MC1, D11 and D7 TCR-like antibodies to normal PBMCs. PBMCs were characterized for HLA-A2 homo or heterozygosity by PCR. Binding of TCR-like antibodies was monitored by PE-labeled secondary anti-mouse IgG.

Figure 18: Summary of D11 TCR-like antibody selectivity. Binding of D11 TCR-like antibodies to HLA-A2+ antigen positive and negative cells was monitored by using PE-labeled anti-mouse IgG. +/- indicate tyrosinase mRNA gene expression as measured by PCR. HLA-A2 expression was monitored with MAb BB7.2.

Figure 19: Summary of D7 TCR-like antibody selectivity. Binding of D7 TCR-like antibodies to HLA-A2+ antigen positive and negative cells was monitored by using PE-labeled anti-mouse IgG. +/- indicate tyrosinase mRNA gene expression as measured by PCR. HLA-A2 expression was monitored with MAb BB7.2.

Figure 20: Binding of MC1, D11, and D7 TCR-like antibodies to T2 APCs loaded with tyrosinase peptide and tyrosinase similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-4} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG.

Figure 21: Binding of D11 TCR-like antibody to T2 APCs loaded with tyrosinase peptide similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 22: Binding of D11 TCR-like antibody to T2 APCs loaded with tyrosinase peptide similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 23: Binding of D11 TCR-like antibody to T2 APCs loaded with tyrosinase peptide similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 24: Binding of D11 TCR-like antibody to T2 APCs loaded with tyrosinase similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 25: Binding of D7 TCR-like antibody to T2 APCs loaded with tyrosinase similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 26: Binding of D7 TCR-like antibody to T2 APCs loaded with tyrosinase similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 27: Binding of D7 TCR-like antibody to T2 APCs loaded with tyrosinase similar HLA-A2 restricted peptides. T2 cells were loaded with Tyrosinase peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 28: Binding of D7 TCR-like antibody to T2 APCs loaded with tyrosinase similar HLA-A2 restricted peptides identified after alanine scanning. T2 cells were loaded with Tyrosinase peptide and indicated peptides which were selected according to epitope recognition specificity of by D7 of Ala mutated peptides at a concentration of 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 29: Apparent binding affinity determination of TCR-like antibody B47B6 targeting HLA-A2/WT1 complexes. Purified IgGs were immobilized indirectly to the SPR sensor chip with anti-mouse. Analyte was purified recombinant single-chain HLA-A2/WT1 complexes generated by in vitro refolding of E.coli expressed scHLA-A2 complexes.

Figure 30: Binding of B47 and ESK1 TCR-like antibodies to T2 APCs loaded with WT1 HLA-A2 restricted peptide. T2 cells were loaded with WT1 at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG (for B47) or human IgG (for ESK1). MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 31: Binding of B47 and ESK1 TCR-like antibodies to T2 APCs loaded with WT1 peptide and control HLA-A2 restricted peptides. T2 cells were loaded with WT1 peptide and indicated peptides at a concentration of 10^{-4} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG (for B47) or human IgG (for ESK1). MAb BB7.2 was used to monitor expression of HLA-A2. Mean fluorescence intensity (MFI) is indicated.

Figure 32: Binding of B47 and ESK1 TCR-like antibodies to T2 APCs loaded with WT1 similar HLA-A2 restricted peptides. T2 cells were loaded with WT1 peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG (for B47) or human IgG (for ESK1). Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 33: Binding of B47 TCR-like antibody to T2 APCs loaded with WT1 peptide or control HLA-A2 restricted peptides. T2 cells were loaded with WT1 peptide and indicated peptides at a concentration of 10^{-4} M for 12 hrs at 37°C. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 34: Binding of B47 TCR-like antibody to T2 APCs loaded with WT1 similar HLA-A2 restricted peptides. T2 cells were loaded with WT1 peptide and indicated peptides at a concentration of 10^{-4} - 10^{-5} M for 12 hrs at 37°C. Binding was

monitored by flow cytometry using secondary PE-labeled anti-mouse IgG. Binding of MAb BB7.2 ensured measurement of peptide loading efficiency.

Figure 35: Binding of B47 and ESK1 TCR-like antibodies to HLA-A2 positive cells that express or not express WT1. Binding was monitored by flow cytometry using secondary PE-labeled anti-mouse IgG (for B47) or human IgG (for ESK1). Expression of HLA-A2 was assessed with MAb BB7.2.

Figure 36: Summary of B47 TCR-like antibody selectivity. Binding of B47 TCR-like antibodies to HLA-A2+ antigen positive and negative cells was monitored by using PE-labeled anti-mouse IgG. +/- indicate WT1 mRNA gene expression as measured by PCR. HLA-A2 expression was monitored with MAb BB7.2.

Figure 37: Epitope specificity determination of TCR-like antibodies by Alanine scanning. The WT1 peptide sequence was substituted with Alanine at positions 1, 3, 4,5, 7, and 8. The Ala mutated peptides were synthesized and loaded APCs Binding of TCR-like antibody ESK1 was accessed by flow cytometry and binding intensity as measured by mean fluorescence intensity was measured and compared with the binding intensity to WT native WT1 peptide. The relative effect of each position Ala substitution was evaluated as percentage to the binding to WT peptide. Data from Dao et al. Sci Transl Med 5, 176ra33 (2013).

Figure 38: Binding of D11, D7, and biotinylated MC1 to T2 APCs loaded with Tyrosinase peptide and Tyrosinase similar HLA-A2 restricted peptides. S17-S23 are Alanine-based similar peptides. T2 cells were loaded with Tyrosinase and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with TCRL antibodies at a concentration of 10 µg/ml followed by secondary PE-labeled streptavidin/anti-mouse antibody and analyzed by flow cytometry Mean fluorescence intensity (MFI) is indicated.

Figure 39: Binding of D11, D7 and MC1 TCR-like antibodies to T2 APCs loaded with Tyrosinase peptide and Tyrosinase similar HLA-A2 restricted peptides. KIAA0355, S7, S17-S23 are Alanine-based similar peptides. T2 cells were loaded with Tyrosinase and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with TCRL antibodies at a concentration of 10 µg/ml followed by secondary PE-labeled streptavidin/anti-mouse antibody and analyzed by flow cytometry Mean fluorescence intensity (MFI) is indicated.

Figures 40A-C: Binding of D11 (Figure 40A), D7 (Figure 40B) and biotinylated MC1 (Figure 40C) TCR-like antibodies to HLA-A2+, Tyrosinase antigen positive or negative cells. Tumor and normal primary cells that express HLA-A2 were tested by qPCR for Tyrosinase mRNA expression. Tumor cells were stained with the indicated
 5 TCR-like antibodies at a concentration of 10 µg/ml followed by secondary PE-labeled streptavidin/anti-mouse antibody and analyzed by flow cytometry. Mean fluorescence intensity (MFI) is indicated.

Figure 41: Killing of HLA-A2+/Tyrosinase+ (positive) and HLA-A2+/Tyrosinase- (negative) cell lines by bi-specific (BS) TCRL having an anti CD-3
 10 arm and a D11 arm, termed Tyr D11 BS TCRL. Tyr D11 BS TCRL was incubated with melanoma HLA-A2+/Tyrosinase+ cells and control tumor cells that are HLA-A2+/Tyrosinase-. Cells were incubated for 24 hrs with the Tyr D11 BS TCRL and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio (10:1 effector:target ratio). Cytotoxicity determined by lactate dehydrogenase (LDH) release assay.

15 Figure 42: Killing of HLA-A2+/Tyrosinase- normal primary cells by Tyr D11. BS D11 was incubated with melanoma HLA-A2+/Tyrosinase+ cells as control and normal primary cells that are HLA-A2+/Tyrosinase-. Cells were incubated for 24 hrs with the D11 BS TCRL and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio.

20 Figure 43: Killing of HLA-A2+/Tyrosinase+ and HLA-A2+/Tyrosinase- cell lines by Tyr D7 BS TCRL. D7 BS was incubated with melanoma HLA-A2+/Tyrosinase+ cells and control tumor cells that are HLA-A2+/Tyrosinase-. Cells were incubated for 24 hrs with the D7 BS and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio.

25 Figure 44: Killing of HLA-A2+/Tyrosinase- normal primary cells by D7 BS. D7 BS was incubated with melanoma HLA-A2+/Tyrosinase+ cells as control and normal primary cells that are HLA-A2+/Tyrosinase-. Cells were incubated for 24 hrs with the D7 BS and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio.

30 Figure 45 In vivo efficacy of D7 BS in preventing an S.C. 501A melanoma tumor formation in NOD/SCID mice.

Figure 46: Binding of biotinylated ESK1 and B47B6 TCR-like antibodies to T2 APCs loaded with WT1 peptide and other HLA-A2 restricted peptides. T2 cells were

loaded with WT1 peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with ESK1 or B47B6 TCRL antibodies at a concentration of 10 µg/ml followed by secondary PE-labeled streptavidin/anti-mouse antibody and analyzed by flow cytometry Mean fluorescence intensity (MFI) is indicated.

5 Figure 47: Binding of ESK1 and B47B6 TCR-like antibodies to T2 APCs loaded with WT1 peptide and WT1 similar HLA-A2 restricted peptides. S2, S6 and S7 are Alanine-based similar peptides. S11 is a heteroclitic peptide. T2 cells were loaded with WT1 peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with ESK1 or B47B6 TCRL antibodies at a concentration of 10
10 µg/ml followed by secondary PE-labeled streptavidin/anti-mouse antibody and analyzed by flow cytometry Mean fluorescence intensity (MFI) is indicated.

Figure 48: Affinity by SPR - Apparent binding affinity determination of ESK1 and B47B6 TCR-like antibodies targeting HLA-A2/WT1 complexes. Purified recombinant biotinylated single-chain HLA-A2/WT1 complex generated by in vitro
15 refolding of E.coli expressed scHLA-A2 complexes, was immobilized indirectly to the SPR sensor chip with NeutrAvidin. Purified ESK1 and B47B6 TCRL Fabs served as analytes.

Figure 49: Epitope specificity determination by Alanine scanning mutagenesis. The mutant WT1 peptides with Alanine substitutions at positions 1, 2, 3, 4, 5, 7, 8 and 9
20 were synthesized and loaded onto T2 cells APCs at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with the B47B6 TCR-like antibody at a concentration of 10 µg/ml and analyzed by flow cytometry. The relative effect of Ala substitution at each position was expressed as percentage of the binding to wild-type peptide.

Figure 50: Binding of ESK1 and B47B6 TCR-like antibodies to HLA-A2+ and
25 WT1 mRNA positive or negative cells. Tumor cells that express HLA-A2 were tested by qPCR for WT1 mRNA expression. Tumor cells were stained with biotinylated ESK1 and B47B6 TCRL antibodies at 10 µg/ml followed by secondary PE-labeled streptavidin. Mean fluorescence intensity (MFI) is indicated. Also shown are mRNA expression data and cell killing with the bispecific forms (with anti-CD3) of the
30 antibodies, as described herein.

Figure 51A: Killing of HLA-A2+/WT1+ and HLA-A2+/WT1- normal primary cells by B47B6 BS vs ESK1 BS. B47B6 BS and ESK1 BS were incubated with normal

primary cells that are HLA-A2+/WT1+ or HLA-A2+/WT1-. Cells were incubated for 24 hrs with the B47B6 BS or ESK1 BS and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio. Cytotoxicity was determined by LDH release assay.

Figure 51B: Killing of HLA-A2+/WT1+ and HLA-A2+/WT1- cell lines by B47B6 BS vs ESK1 BS. B47B6 BS and ESK1 BS were incubated with tumor cells that are HLA-A2+/WT1+ or HLA-A2+/WT1-. Cells were incubated for 24 hrs with the B47B6 BS or ESK1 BS and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio (#F3-Format - in which the anti-CD3 scFv fragment was fused to the VLCL of the Fab).

Figure 52: Binding of C106B9 TCR-like antibody to T2 APCs loaded with MAGE-A4₂₃₀₋₂₃₉ (also referred to as MAGE-A4 peptide) peptide and other HLA-A2 restricted peptides. T2 cells were loaded with MAGE-A4 and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with C106B9 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody and analyzed by flow cytometry. Mean fluorescence intensity (MFI) is indicated.

Figure 53: Binding of C106B9 TCR-like antibody to T2 APCs loaded with MAGE-A4 peptide and MAGE-A4 similar HLA-A2 restricted peptides. T2 cells were loaded with MAGE-A4 and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with C106B9 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody and analyzed by flow cytometry.

Figure 54: Affinity by SPR - Apparent binding affinity determination of C106B9 TCR-like antibody targeting HLA-A2/MAGE-A4 complexes. Purified recombinant biotinylated single-chain HLA-A2/MAGE-A4 complex generated by in vitro refolding of E.coli expressed scHLA-A2 complexes, was immobilized indirectly to the SPR sensor chip with NeutrAvidin. Purified C106B9 TCRL Fab was used as the analyte.

Figure 55: Epitope specificity determination by Alanine scanning mutagenesis. The mutant MAGE-A4 peptides with alanine substitutions at positions 1,2,3,4,5,6,7,8 and 9 were synthesized. Possible anchor positions are shown by a gray star. The native and mutant MAGE-A4 peptides were loaded onto T2 cells APCs at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with C106B9 TCR-like antibody at a concentration of 10µg/ml and analyzed by flow cytometry. MFI values for cells loaded

with mutant and wild type peptides were compared. The relative effect of each Ala substitution was expressed as percentage of the binding to native wild-type peptide.

Figure 56: Binding of C106B9 TCR-like antibody to HLA-A2+ and MAGE-A4 antigen positive or negative cells. Expression of MAGE-A4 mRNA in the cells was confirmed by qPCR. Tumor cells were stained with C106B9 at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody and analyzed by flow cytometry. Mean fluorescence intensity (MFI) is indicated. Also shown are mRNA expression data and cell killing with the bispecific forms (with anti-CD3) of the antibodies, as described herein.

Figure 57: Killing of HLA-A2+/MAGE-A4+ and HLA-A2+/MAGE-A4- cell lines by C106B9 BS. C106B9 BS was incubated with tumor cells that are HLA-A2+/MAGE-A4+ cells and control tumor cells that are HLA-A2+/MAGE-A4-. Cells were incubated for 24 hrs with the C106B9 BS and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio.

Figure 58: Killing of HLA-A2+/MAGE-A4- normal primary cells by C106B9 BS. C106B9 BS was incubated with normal primary cells that are HLA-A2+/MAGE-A4-. Cells were incubated for 24 hrs with the C106B9 BS and with naïve PBMCs isolated from healthy individuals at 10:1 E:T ratio.

Figure 59: In vivo efficacy of MAGE-A4 BS C106B9 BS in prevention of S.C. melanoma tumor formation in NOD/SCID mice.

Figure 60: Binding of F184C7 TCR-like antibody to T2 APCs loaded with MAGE-A9₂₂₃₋₂₃₁ peptide (also referred to as MAGE-A9 peptide) and other HLA-A2 restricted peptides. T2 cells were loaded with MAGE-A9 peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with F184C7 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody and analyzed by flow cytometry. Mean fluorescence intensity (MFI) is indicated.

Figure 61: Binding of F184C7 TCR-like antibodies to T2 APCs loaded with MAGE-A9 peptide and MAGE-A9 similar HLA-A2 restricted peptides. S8 is an Alanine-based similar peptide. T2 cells were loaded with MAGE-A9 peptide and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained

with F184C7 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody and analyzed by flow cytometry.

Figure 62: Epitope specificity determination by Alanine scanning mutagenesis. The mutant MAGE-A9 peptides with alanine substitutions at positions 2,3,4,5,6,7,8 and 9 were synthesized. The Ala mutant and native peptides were loaded onto T2 cells APCs at a concentration of 10^{-5} M for 12 hrs at 37°C. Cells were stained with F184C7 TCR-like antibody at a concentration of 10µg/ml and analyzed by flow cytometry. MFI values for cells loaded with mutant and wild type peptides were compared. The relative effect of each Ala substitution was expressed as percentage of the binding to native peptide.

Figure 63: Binding of F184C7 TCR-like antibody to HLA-A2+ normal primary cells. Normal primary cells were stained with F184C7 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody. Mean fluorescence intensity (MFI) is indicated.

Figure 64: Binding of D10A3 TCR-like antibody to T2 APCs loaded with PAP₁₁₂₋₁₂₀ peptide (also referred to as PAP peptide) and other HLA-A2 restricted peptides. T2 cells were loaded with PAP and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with D10A3 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody. Mean fluorescence intensity (MFI) is indicated.

Figure 65: Binding of D10A3 TCR-like antibodies to T2 APCs loaded with PAP peptide and PAP similar HLA-A2 restricted peptides. T2 cells were loaded with PAP and indicated peptides at a concentration of 10^{-5} M for 12 hrs at 37 °C. Cells were stained with D10A3 TCRL antibody at 10 µg/ml followed by secondary PE-labeled anti-mouse antibody. Mean fluorescence intensity (MFI) is indicated.

Figure 66: Epitope specificity determination by Alanine scanning mutagenesis. The mutant PAP peptides with Alanine substitutions at positions 1,3,4,6,7,8 and 9 were synthesized and loaded onto T2 cells APCs at a concentration of 10^{-5} M for 12 hrs at 37°C. Cells were stained with D10A3 TCR-like antibody at a concentration of 10µg/ml. MFI values for cells loaded with mutant and wild type peptides were compared. The relative effect of each Ala substitution was expressed as percentage of the binding to WT peptide.

Figure 67: Binding of D10A3 TCR-like antibody to HLA-A2+ normal primary cells. Normal primary cells were stained with D10A3 TCRL antibody at 10µg/ml followed by secondary PE-labeled anti-mouse antibody. Mean fluorescence intensity (MFI) is indicated.

5 Figure 68: Amino acids and nucleic acids of D11 antibody (SEQ ID NOs: 280-295).

Figure 69: Amino acids and nucleic acids of D7 antibody (SEQ ID NOs: 296-311).

Figure 70: Amino acids and nucleic acids of B47B6 antibody (SEQ ID NOs: 312-327).

Figure 71: Amino acids and nucleic acids of C106B9 antibody (SEQ ID NOs: 328-343).

Figure 72: Amino acids and nucleic acids of F184C7 antibody (SEQ ID NOs: 344-359).

15 Figure 73: Amino acids and nucleic acids of D10A3 antibody (SEQ ID NOs: 360-375).

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to affinity entities comprising a TCR-like antibody binding domain with affinity and fine specificity and uses of same.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

25 T Cell Receptor (TCR)-like (TCRL) antibodies are endowed with a TCR-like specificity toward tumor epitopes. Unlike TCRs which exhibit low affinity to the MHC-peptide antigen complex, TCRLs are characterized by affinity even at their soluble form. TCRLs are being developed as a new therapeutic class for targeting tumor cells and mediating their specific killing. In addition, these antibodies are valuable research reagents enabling the study of human class I peptide-MHC ligand presentation and TCR-peptide-MHC interactions.

The present inventors have now indentified through a laborious screen and experimentation novel TCRLs which exhibit unprecedented fine specificity towards TyrD-HLA-A2 (D7 and D11), WT1-HLA-A2 (B47), MAGE-A4-HLA-A2 (C106B9), MAGE-A9-HLA-A2 (F184C7) and PAP (D10A3). The CDRs of these antibodies can
5 be implanted in any affinity binding entity such as having an effector activity e.g., a CAR and TCR.

Thus, according to an aspect of the invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 Heavy Chain (HC)	SEQ ID NO: 309	SYGVH
CDR2 HC	SEQ ID NO: 310	VIWAGGTTNYSALMS
CDR3 HC	SEQ ID NO: 311	DGHFHFDF
CDR1 Light Chain (LC)	SEQ ID NO: 303	RASDIIYSNLA
CDR2 LC	SEQ ID NO: 304	AATNLAA
CDR3 LC	SEQ ID NO: 305	QHFWGSSIS

10 the affinity binding entity capable of binding HLA-A2/Tyr_{D369-377} in an MHC restricted manner.

According to an aspect of the invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 Heavy Chain (HC)	SEQ ID NO: 293	TSGMGVS
CDR2 HC	SEQ ID NO: 294	HIYWDDDKRYNPSLKS
CDR3 HC	SEQ ID NO: 295	KDYGSSFYAMHY
CDR1 Light Chain (LC)	SEQ ID NO: 287	KASQDIHNYIA
CDR2 LC	SEQ ID NO: 288	YTSTLQP
CDR3 LC	SEQ ID NO: 289	LQYDNLWT

15 the affinity binding entity capable of binding HLA-A2/Tyr_{D369-377} in an MHC restricted manner.

According to an aspect of the invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 325	SYDMS
CDR2 HC	SEQ ID NO: 326	YMSSGGGTYPDTVKG
CDR3 HC	SEQ ID NO: 327	HDEITNFDY

CDR1 LC	SEQ ID NO: 319	RASQSI NSLH
CDR2 LC	SEQ ID NO: 320	YASQSI
CDR3 LC	SEQ ID NO: 321	QQSYSWPLT

the affinity binding entity capable of binding HLA-A2/ WT1₁₂₆₋₁₃₄ in an MHC restricted manner.

According to an aspect of the invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-

5 C ordered:

CDR1 HC	SEQ ID NO: 341	GYWIE
CDR2 HC	SEQ ID NO: 342	EILPGSGGTNYNEKFKG
CDR3 HC	SEQ ID NO: 343	DSNSFTY
CDR1 LC	SEQ ID NO: 335	SVSSSVDYIH
CDR2 LC	SEQ ID NO: 336	STSILAS
CDR3 LC	SEQ ID NO: 337	QQRSSYT

the affinity binding entity capable of binding HLA-A2/ MAGE-A4₃₂₈₋₃₄₃ in an MHC restricted manner.

According to an aspect of the invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-

10 C ordered:

CDR1 HC	SEQ ID NO: 357	FSSSWMN
CDR2 HC	SEQ ID NO: 358	RIYPGDGDTNYNEKFKG
CDR3 HC	SEQ ID NO: 359	EATTVVAPYYFDY
CDR1 LC	SEQ ID NO: 351	RASENIYRNLA
CDR2 LC	SEQ ID NO: 352	AATNLAD
CDR3 LC	SEQ ID NO: 353	QHFWGTPLT

the affinity binding entity capable of binding HLA-A2/ MAGE-A9₃₄₄₋₃₅₉ in an MHC restricted manner.

According to an aspect of the invention there is provided an affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-

15 C ordered:

CDR1 HC	SEQ ID NO: 373	DYNMD
CDR2 HC	SEQ ID NO: 374	DINPNYDTTTYNQKFKG
CDR3 HC	SEQ ID NO: 375	RNYGNYVGDFD
CDR1 LC	SEQ ID NO: 367	KASQRVNNDVA
CDR2 LC	SEQ ID NO: 368	YASNRYT
CDR3 LC	SEQ ID NO: 369	QQDYSSPFT

the affinity binding entity capable of binding HLA-A2/ PAP₃₆₀₋₃₇₅ in an MHC restricted manner.

As used herein a "T Cell Receptor-like antibody" or "TCRL" refers to an antibody which binds an MHC being complexed with an HLA-restricted peptide antigen. Binding of the TCRL to its target is with an MHC-restricted specificity. The TCRL antibody does not bind said MHC in the absence of said complexed peptide, and the antibody does not bind said peptide in an absence of said MHC.

As used herein "binding" or "binds" refers to an antibody-antigen mode of binding, which is generally, in the case of clinically relevant TCRLs, in the range of K_D below 20 nM, as determined by Surface Plasmon Resonance assay (SPR).

The affinity of the antigen binding domain to its antigen is determined using the soluble form of the antibody from which the CDRs of the antigen binding domain of the antibody are derived. For affinity evaluation, the antigen is used in its soluble form e.g., as a single chain MHC-peptide complex as further described hereinbelow.

As used herein the term " K_D " refers to the equilibrium dissociation constant between the antigen binding domain and its respective antigen.

It will be appreciated that the affinity of the affinity binding entity is determined by the CDRs. However, the affinity may be improved using methods known in the art, such as affinity maturation.

As used herein "affinity binding entity" refers to a binding moiety which binds to a specific antigen with a higher affinity than to a non-specific antigen and is endowed with an affinity of at least 10^{-6} M, as determined by assays which are well known in the art, including SPR.

According to a specific embodiment the affinity is 500 nM- 0.5 nM, 100 nM-1 nM, 50 nM-1 nM, 20 nM-1 nM, 10 nM-1 nM.

The affinity moiety may be selected from the group consisting of TCR, CAR-T and an antibody.

According to a specific embodiment, the affinity binding entity is an antibody. Although the reference to antibodies is in more details as compared to other affinity binding entities, the description of this embodiment should not be construed as limiting and the present invention is equally related to binding entities as described herein especially in the sense of cell therapy as further described hereinbelow.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')₂, Fv, scFv, dsFv, or single domain

molecules such as VH and VL that are capable of binding to an epitope of an antigen in an MHC restricted manner. As a more general statement the term “antibody” aims to encompass any affinity binding entity which binds a cell surface presented molecule with an MHC restricted specificity. Thus, CDRs of the antibodies of some
 5 embodiments of the present invention may be implanted in artificial molecules such as T cell receptors or CARs as further described hereinbelow.

Suitable antibody fragments for practicing some embodiments of the invention include a complementarity-determining region (CDR) of an immunoglobulin light chain (referred to herein as “light chain”), a complementarity-determining region of an
 10 immunoglobulin heavy chain (referred to herein as “heavy chain”), a variable region of a light chain, a variable region of a heavy chain, a light chain, a heavy chain, an Fd fragment, and antibody fragments comprising essentially whole variable regions of both light and heavy chains such as an Fv, a single chain Fv Fv (scFv), a disulfide-stabilized Fv (dsFv), an Fab, an Fab’, and an F(ab’)2.

As used herein, the terms "complementarity-determining region" or "CDR" are used interchangeably to refer to the antigen binding regions found within the variable region of the heavy and light chain polypeptides. Generally, antibodies comprise three CDRs in each of the VH (CDR HI or HI; CDR H2 or H2; and CDR H3 or H3) and three in each of the VL (CDR LI or LI; CDR L2 or L2; and CDR L3 or L3). Examples of
 15 such CDR sequences are provide for D7 and D11 – TCRLs produced according to Example I below. Additional examples include, WT1 B47B6, MAGE-A4 C106B9, MAGE-A9 F184C7, PAP D10A3 (shown in Figures 68-73).

The identity of the amino acid residues in a particular antibody that make up a variable region or a CDR can be determined using methods well known in the art and
 25 include methods such as sequence variability as defined by Kabat et al. (See, e.g., Kabat et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C.), location of the structural loop regions as defined by Chothia et al. (see, e.g., Chothia et al., Nature 342:877-883, 1989.), a compromise between Kabat and Chothia using Oxford Molecular's AbM antibody modeling software
 30 (now Accelrys®, see, Martin et al., 1989, Proc. Natl Acad Sci USA. 86:9268; and world wide web site www.bioinf-org.uk/abs), available complex crystal structures as defined by the contact definition (see MacCallum et al., J. Mol. Biol. 262:732-745, 1996), the

"conformational definition" (see, e.g., Makabe et al., Journal of Biological Chemistry, 283:1156-1166, 2008) and IMGT [Lefranc MP, et al. (2003) IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. Dev Comp Immunol 27: 55-77].

5 As used herein, the "variable regions" and "CDRs" may refer to variable regions and CDRs defined by any approach known in the art, including combinations of approaches. According to a specific embodiment, the CDRs are determined according to Kabat et al. (supra).

Functional antibody fragments comprising whole or essentially whole variable
10 regions of both light and heavy chains are defined as follows:

(i) Fv, defined as a genetically engineered fragment consisting of the variable region of the light chain (VL) and the variable region of the heavy chain (VH) expressed as two chains;

(ii) single chain Fv ("scFv"), a genetically engineered single chain molecule
15 including the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule;

(iii) disulfide-stabilized Fv ("dsFv"), a genetically engineered antibody
20 including the variable region of the light chain and the variable region of the heavy chain, linked by a genetically engineered disulfide bond;

(iv) Fab, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme papain to yield the intact light chain and the Fd fragment of the heavy chain which consists of the variable and CH1 domains thereof;

25 (v) Fab', a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme pepsin, followed by reduction (two Fab' fragments are obtained per antibody molecule);

(vi) F(ab')₂, a fragment of an antibody molecule containing a monovalent
30 antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme pepsin (i.e., a dimer of Fab' fragments held together by two disulfide bonds); and

(vii) Single domain antibodies or nanobodies are composed of a single VH or VL domains which exhibit sufficient affinity to the antigen.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to some embodiments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (1972)]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments

comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, *Methods* 2: 97-105 (1991); Bird et al., *Science* 242:423-426 (1988); Pack et al., *Bio/Technology* 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [*Methods*, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region

(Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art.

5 Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*
10 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice,
15 humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381
20 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*, 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice
25 in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following
30 scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368 812-13 (1994); Fishwild et al.,

Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

In an embodiment in which the antibody is a full length antibody, the heavy and light chains of an antibody of the invention may be full-length (e.g., an antibody can include at least one, and preferably two, complete heavy chains, and at least one, or two, complete light chains) or may include an antigen-binding portion (a Fab, F(ab').sub.2, Fv or a single chain Fv fragment ("scFv")). In other embodiments, the antibody heavy chain constant region is chosen from, e.g., IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD, and IgE. In some embodiments, the immunoglobulin isotype is selected from IgG1, IgG2, IgG3, and IgG4, more particularly, IgG1 (e.g., human IgG1) or IgG4 (e.g., human IgG4). The choice of antibody type will depend on the immune effector function that the antibody is designed to elicit.

Bispecific configurations of antibodies are also contemplated herein. A bispecific monoclonal antibody (BsMAb, BsAb) is an artificial protein that is composed of fragments of two different monoclonal antibodies and consequently binds to two different types of antigen. According to a specific embodiment the BsMAb is engineered to simultaneously bind to a cytotoxic cell (e.g., using a receptor like CD3) and a target like a tumor cell to be destroyed (further described hereinbelow).

As used herein the phrase "chimeric antigen receptor (CAR)" refers to a recombinant or synthetic molecule which combines antibody-based specificity for a desired antigen with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits cellular immune activity to the specific antigen.

As used herein the phrase "T Cell Receptor" or "TCR" refers to soluble and non-soluble forms of recombinant T cell receptor.

As used herein the phrase "MHC (or HLA)-restricted peptide" refers to a peptide which is potentially presented on an MHC molecule. Such peptides may be identified by "wet" laboratory procedures such as Mass-Spectrometry or by *in-silico* analysis. An MHC (or HLA)-presented peptide refers to a peptide which is confirmed in vitro or in vivo as being presented by an MHC molecule.

According to a specific embodiment, the MHC restricted peptide is from WT1 and the affinity binding entity comprises the CDRs of B47B6.

According to a specific embodiment, the MHC restricted peptide is from TyrD and the affinity binding entity comprises the CDRs of D7 or D11.

According to a specific embodiment, the MHC restricted peptide is from MAGE-A4 and the affinity binding entity comprises the CDRs of C106B9.

5 According to a specific embodiment, the MHC restricted peptide is from MAGE-A9 and the affinity binding entity comprises the CDRs of F184C7.

According to a specific embodiment, the MHC restricted peptide is from PAP and the affinity binding entity comprises the CDRs of D10A3.

10 CDRs of the above mentioned affinity binding entities are described in Figures 68-73.

Also contemplated are homologous sequences e.g., at least 90 % homology, 95 % homology or even at least 99 % homology as long as the binding affinity to the respective target and optionally specificity are maintained or even improved.

15 According to an aspect of the invention there is also provided an isolated polynucleotide comprising a nucleic acid sequence encoding the affinity binding entity as described herein.

Also provided is an expression vector, comprising the polynucleotide operably linked to a cis- acting regulatory element.

20 The nucleic acid construct (also referred to herein as an "expression vector") of some embodiments of the invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, typical cloning vectors may also contain a transcription and translation initiation sequence, transcription and translation terminator and a polyadenylation signal. By way of example, such constructs will typically include
25 a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof.

The nucleic acid construct of some embodiments of the invention typically includes a signal sequence for secretion or presentation of the affinity binding entity from a host cell in which it is placed. Preferably the signal sequence for this purpose is
30 a mammalian signal sequence.

Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs

upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.

Preferably, the promoter utilized by the nucleic acid construct of some
5 embodiments of the invention is active in the specific cell population transformed. Examples of cell type-specific and/or tissue-specific promoters include promoters such as albumin that is liver specific [Pinkert et al., (1987) *Genes Dev.* 1:268-277], lymphoid specific promoters [Calame et al., (1988) *Adv. Immunol.* 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) *EMBO J.* 8:729-733] and
10 immunoglobulins; [Banerji et al. (1983) *Cell* 33:729-740], neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477], pancreas-specific promoters [Edlunh et al. (1985) *Science* 230:912-916] or mammary gland-specific promoters such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166).

Enhancer elements can stimulate transcription up to 1,000 fold from linked
15 homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter
20 combinations that are suitable for some embodiments of the invention include those derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, *Enhancers and Eukaryotic Expression*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by
25 reference.

In the construction of the expression vector, the promoter is preferably positioned approximately the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter
30 function.

Polyadenylation sequences can also be added to the expression vector in order to increase the efficiency of TCRL mRNA translation. Two distinct sequence elements

are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for some embodiments of the invention include those derived from SV40.

In addition to the elements already described, the expression vector of some embodiments of the invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The vector may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

Also provided are cells which comprise the polynucleotides/expression vectors as described herein.

Such cells are typically selected for high expression of recombinant proteins (e.g., bacterial, plant or eukaryotic cells e.g., CHO, HEK-293 cells), but may also be host cells having a specific immune effector activity (e.g., T cells or NK cells) when for instance the CDRs of the TCRL are implanted in a T Cell Receptor or CAR transduced in said cells which are used in adoptive cell therapy as further described hereinbelow.

The high specificity of the affinity binding entity renders it particularly suitable for diagnostic and therapeutic applications.

Thus, according to an aspect of the present invention, there is provided a method of detecting a cell presenting an HLA-restricted peptide antigen of interest. The method comprises contacting the cell with the affinity binding entity (e.g., antibody) of the present invention having specificity to the HLA-restricted peptide antigen of interest.

The contacting is effected under conditions which allow immunocomplex formation, wherein a presence of the immunocomplex or level thereof is indicative of the cell presenting the HLA-restricted peptide antigen of interest.

The term “detecting”, as used herein, refers to the act of detecting, perceiving, uncovering, exposing, visualizing or identifying a cell. The precise method of detecting is dependent on the detectable moiety (also referred to herein as identifiable moiety) to which the antibody is attached as further described herein below.

Single cells may be used in accordance with the teachings of the present invention as well as a plurality of cells. For instance the cells may be from any biological sample such as cell-lines, primary (e.g., tumor cultures) and cellular samples, e.g. surgical biopsies including incisional or excisional biopsy, fine needle aspirates and the like. Methods of biopsy retrieval are well known in the art.

The above-mentioned detection method can be harnessed to the diagnosis of diseases which are characterized by above normal presentation or different tissue distribution of the HLA-peptide complex.

As used herein the term "diagnosing" refers to classifying a disease, determining a severity of a disease (grade or stage), monitoring progression, forecasting an outcome of the disease and/or prospects of recovery.

The subject may be a healthy subject (e.g., human) undergoing a routine well-being check up. Alternatively, the subject may be at risk of the disease. Yet alternatively, the method may be used to monitor treatment efficacy.

The TCRL may comprise e.g., attached to an identifiable moiety. Alternatively or additionally, the TCRL (or a complex comprising same) may be identified indirectly such as by using a secondary antibody.

The contacting may be effected in vitro (i.e. in a cell line, primary cells), ex vivo or in vivo.

As mentioned, the method of the present invention is effected under conditions sufficient to form an immunocomplex (e.g. a complex between the antibodies of the present invention and the peptide complexed to the MHC, typically when the cells are not lysed); such conditions (e.g., appropriate concentrations, buffers, temperatures, reaction times) as well as methods to optimize such conditions are known to those skilled in the art, and examples are disclosed herein.

The affinity binding entities of the invention (e.g., antibodies) are especially useful for the treatment of cancer.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the
5 bloodstream and lymphatic system to other parts of the body.

The cancer may be a hematological malignancy, a solid tumor, a primary or a metastasizing tumor. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, Chronic
10 Lymphocytic Leukemia (CLL), leukemia, lung cancer and the like. Additional non-limiting examples of cancers which can be treated by the method of some embodiments of the invention are provided in Table 1, above.

Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise
15 non-solid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the Antibodies of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and
20 pediatric tumors/cancers are also included.

Hematologic cancers are cancers of the blood or bone marrow. Examples of hematological (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and
25 erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and
30

lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma and brain metastases).

According to some embodiments of the invention, the pathology is a solid tumor.

According to some embodiments of the invention, the affinity binding entity of the invention has an anti-tumor effect.

The term "anti-tumor effect" as used herein, refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An "anti-tumor effect" can also be manifested by the ability of the medicament of the invention in prevention of the occurrence of tumor in the first place.

According to a specific embodiment, when the affinity binding entity is for Tyrosinase (TyrD) the cancer is selected from the group consisting of melanoma and glioblastoma.

According to a specific embodiment, when the affinity binding entity is for WT1 the cancer is selected from:

Table 1

Leukemia
multiple myeloma (MM)
acute lymphoblastic leukemia (ALL)
acute myeloid/myelogenous leukemia (AML)
myelodysplastic syndrome (MDS)
mesothelioma
ovarian cancer
gastrointestinal cancers e.g., colorectal cancer adenocarcinoma, thyroid cancer
breast cancer
lung cancer (e.g., non small cell lung cancer)
melanoma
osteosarcoma
endometrial cancer

According to a specific embodiment, when said affinity binding entity is for MAGE said cancer is selected from:

5

Table 2

MAGE-A4
Ovarian cancer
T cell leukemia/lymphoma (e.g., ATLL)
Sarcoma
testicular cancer
head and neck cancer
bladder cancer
esophagus cancer.

Table 3

MAGE-A9
renal cell carcinoma
bladder cancer
breast cancer
hepatocellular carcinoma.

According to a specific embodiment, when said affinity binding entity is for PAP said cancer is prostate cancer.

10

The foregoing classifications are relevant for both diagnosis and treatment.

Determining a presence or level of the immunocomplex of the present invention is dependent on the detectable moiety to which the antibody is attached.

15

Examples of detectable moieties that can be used in the present invention include but are not limited to radioactive isotopes, phosphorescent chemicals, chemiluminescent chemicals, fluorescent chemicals, enzymes, fluorescent polypeptides and epitope tags. The detectable moiety can be a member of a binding pair, which is identifiable via its interaction with an additional member of the binding pair, and a label

which is directly visualized. In one example, the member of the binding pair is an antigen which is identified by a corresponding labeled antibody. In one example, the label is a fluorescent protein or an enzyme producing a colorimetric reaction.

Further examples of detectable moieties, include those detectable by Positron
5 Emission Tomography (PET) and Magnetic Resonance Imaging (MRI), all of which are well known to those of skill in the art.

When the detectable moiety is a polypeptide, the immunolabel (i.e. the antibody conjugated to the detectable moiety) may be produced by recombinant means or may be chemically synthesized by, for example, the stepwise addition of one or more amino
10 acid residues in defined order using solid phase peptide synthetic techniques. Examples of polypeptide detectable moieties that can be linked to the antibodies of the present invention using recombinant DNA technology (in which the polynucleotide encoding the TCRL is translationally fused to the detectable moiety) include fluorescent polypeptides, phosphorescent polypeptides, enzymes and epitope tags.

15 Alternatively, chemical attachment of a detectable moiety to the antibodies of the present invention can be effected using any suitable chemical linkage, direct or indirect, as via a peptide bond (when the detectable moiety is a polypeptide), or via covalent bonding to an intervening linker element, such as a linker peptide or other chemical moiety, such as an organic polymer. Such chimeric peptides may be linked
20 via bonding at the carboxy (C) or amino (N) termini of the peptides, or via bonding to internal chemical groups such as straight, branched or cyclic side chains, internal carbon or nitrogen atoms, and the like. Such modified peptides can be easily identified and prepared by one of ordinary skill in the art, using well known methods of peptide synthesis and/or covalent linkage of peptides. Description of fluorescent labeling of
25 antibodies is provided in details in U.S. Pat. Nos. 3,940,475, 4,289,747, and 4,376,110.

Exemplary methods for conjugating two peptide moieties are described herein below:

SPDP conjugation:

Any SPDP conjugation method known to those skilled in the art can be used.
30 For example, in one illustrative embodiment, a modification of the method of Cumber et al. (1985, Methods of Enzymology 112: 207-224) as described below, is used.

A peptide, such as an identifiable or therapeutic moiety, (1.7 mg/ml) is mixed with a 10-fold excess of SPDP (50 mM in ethanol) and the antibody is mixed with a 25-fold excess of SPDP in 20 mM sodium phosphate, 0.10 M NaCl pH 7.2 and each of the reactions incubated, e.g., for 3 hours at room temperature. The reactions are then
5 dialyzed against PBS.

The peptide is reduced, e.g., with 50 mM DTT for 1 hour at room temperature. The reduced peptide is desalted by equilibration on G-25 column (up to 5 % sample/column volume) with 50 mM KH_2PO_4 pH 6.5. The reduced peptide is combined with the SPDP-antibody in a molar ratio of 1:10 antibody:peptide and incubated at 4 °C
10 overnight to form a peptide-antibody conjugate.

Glutaraldehyde conjugation:

Conjugation of a peptide (e.g., an identifiable or therapeutic moiety) with an antibody can be accomplished by methods known to those skilled in the art using glutaraldehyde. For example, in one illustrative embodiment, the method of conjugation
15 by G.T. Hermanson (1996, "Antibody Modification and Conjugation, in Bioconjugate Techniques, Academic Press, San Diego) described below, is used.

The antibody and the peptide (1.1 mg/ml) are mixed at a 10-fold excess with 0.05 % glutaraldehyde in 0.1 M phosphate, 0.15 M NaCl pH 6.8, and allowed to react for 2 hours at room temperature. 0.01 M lysine can be added to block excess sites. After-the
20 reaction, the excess glutaraldehyde is removed using a G-25 column equilibrated with PBS (10 % v/v sample/column volumes).

Carbodiimide conjugation:

Conjugation of a peptide with an antibody can be accomplished by methods known to those skilled in the art using a dehydrating agent such as a carbodiimide. Most
25 preferably the carbodiimide is used in the presence of 4-dimethyl aminopyridine. As is well known to those skilled in the art, carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of peptide and an hydroxyl group of an antibody (resulting in the formation of an ester bond), or an amino group of an antibody (resulting in the formation of an amide bond) or a sulfhydryl group of an antibody
30 (resulting in the formation of a thioester bond).

Likewise, carbodiimide coupling can be used to form analogous covalent bonds between a carbon group of an antibody and an hydroxyl, amino or sulfhydryl group of

the peptide. See, generally, J. March, *Advanced Organic Chemistry: Reaction's, Mechanism, and Structure*, pp. 349-50 & 372-74 (3d ed.), 1985. By means of illustration, and not limitation, the peptide is conjugated to an antibody via a covalent bond using a carbodiimide, such as dicyclohexylcarbodiimide. See generally, the methods of conjugation by B. Neises et al. (1978, *Angew Chem., Int. Ed. Engl.* 17:522; A. Hassner et al. (1978, *Tetrahedron Lett.* 4475); E.P. Boden et al. (1986, *J. Org. Chem.* 50:2394) and L.J. Mathias (1979, *Synthesis* 561). The level of immunocomplex may be compared to a control sample from a non-diseased subject, wherein an up-regulation of immunocomplex formation is indicative of melanoma. Preferably, the subject is of the same species e.g. human, preferably matched with the same age, weight, sex etc. It will be appreciated that the control sample may also be of the same subject from a healthy tissue, prior to disease progression or following disease remission.

According to a specific embodiment, the detection is effected by FACS.

As mentioned the antibodies of the present invention can also be used in therapeutics where the affinity binding entity e.g., antibody comprises a therapeutic moiety.

The therapeutic moiety can be an integral part of the antibody e.g., in the case of a whole antibody, the Fc domain, which activates antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC is a mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies. It is one of the mechanisms through which antibodies, as part of the humoral immune response, can act to limit and contain infection. Classical ADCC is mediated by natural killer (NK) cells; macrophages, neutrophils and eosinophils can also mediate ADCC. For example, eosinophils can kill certain parasitic worms known as helminths through ADCC mediated by IgE. ADCC is part of the adaptive immune response due to its dependence on a prior antibody response.

Alternatively or additionally, the antibody may be a bispecific antibody in which the therapeutic moiety is a T cell engager for example, such as an anti CD3 antibody or an anti CD16a alternatively the therapeutic moiety may be an anti immune checkpoint molecule (anti PD-1).

Alternatively or additionally the antibody may be attached to a heterologous therapeutic moiety (methods of conjugation are described hereinabove). The therapeutic moiety can be, for example, a cytotoxic moiety, a toxic moiety, a cytokine moiety, a drug.

5 The antibody may be in a soluble or insoluble form.

Insoluble forms may be those in which a molecule comprising the antibody's CDRs is anchored to or expressed by a cell or a particle (the latter can be used for therapeutic as well as diagnostic applications).

10 Examples of such cells include immune cells, T cells, B cells, dendritic cells, CIK, NKT, NK cells (autologous, allogeneic, xenogeneic).

According to a specific embodiment, the antibody (or actually CDRs thereof) form a CAR (as explained above) or an artificial T Cell Receptor. Thus a polynucleotide coding for such a molecule is transduced in a cell of interest.

15 According to some embodiments of the invention, the cell is a T cell, a natural killer cell, a cell that exerts effector killing function on a target cell, a cell that exerts a suppressive effect on effector T cells, an engineered cell with an effector killing function or an engineered cell with a suppressive function.

According to some embodiments of the invention, the cell is a T cell, or $\alpha\beta$ T cell, or $\gamma\delta$ T cell.

20 According to some embodiments of the invention, the cell is a natural killer (NK) cell.

According to some embodiments of the invention, the natural killer cell is used to target cancer.

25 According to some embodiments of the invention, the T cell is a cytotoxic T cell (effector T cell).

According to some embodiments of the invention, the cytotoxic T cell (effector T cell) is used to target cancer antigens.

According to some embodiments of the invention, the cytotoxic T cell is used to treat a pathology caused by or associated with cancer.

30 According to some embodiments of the invention, the T cell comprises a Treg (T regulatory cell).

According to some embodiments of the invention, the T cell comprises a CD3 T cell.

According to some embodiments of the invention, the T cell comprises a CD4 T cell.

5 According to some embodiments of the invention, the T cell comprises a CD8 T cell.

According to some embodiments of the invention, the antigen binding domain comprises a single chain Fv (scFv) molecule.

10 The cytoplasmic domain (also referred to as "intracellular signaling domain") of the CAR molecule of the invention is responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been placed in.

The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term "intracellular signaling domain" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

25 Examples of intracellular signaling domains for use in the CAR molecule of the invention include the cytoplasmic sequences of the T cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any synthetic sequence that has the same functional capability.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be mediated by two distinct classes of cytoplasmic signaling sequence: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences) and those that act in an antigen-independent

manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences).

Primary cytoplasmic signaling sequences regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs).

Examples of ITAM containing primary cytoplasmic signaling sequences that are of particular use in the invention include those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. It is particularly preferred that cytoplasmic signaling molecule in the CAR of the invention comprises a cytoplasmic signaling sequence derived from CD3 zeta.

In a preferred embodiment, the cytoplasmic domain of the CAR can be designed to comprise the CD3-zeta signaling domain by itself or combined with any other desired cytoplasmic domain(s) useful in the context of the CAR of the invention. For example, the cytoplasmic domain of the CAR can comprise a CD3 zeta chain portion and a costimulatory signaling region. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. A co-stimulatory molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. Thus, while the invention is exemplified primarily with 4-1BB as the co-stimulatory signaling element, other costimulatory elements are within the scope of the invention.

According to some embodiments of the invention, the intracellular domain comprises, a co-stimulatory signaling region and a zeta chain portion. The co-stimulatory signaling region refers to a portion of the CAR molecule comprising the intracellular domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or their ligands that are required for an efficient response of lymphocytes to antigen.

"Co-stimulatory ligand," as the term is used herein, includes a molecule on an antigen presenting cell [e.g., an aAPC (artificial antigen presenting cell), dendritic cell,

B cell, and the like] that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A co-stimulatory ligand can include, but is not limited to, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, HVEM, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the T cell, such as, but not limited to, proliferation. Co-stimulatory molecules include, but are not limited to an MHC class 1 molecule, BTLA and a Toll ligand receptor.

A "co-stimulatory signal", as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation and/or upregulation or down regulation of key molecules.

By the term "stimulation," is meant a primary response induced by binding of a stimulatory molecule (e.g., a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex. Stimulation can mediate altered expression of certain molecules, such as downregulation of TGF- β , and/or reorganization of cytoskeletal structures, and the like.

A "stimulatory molecule," as the term is used herein, means a molecule on a T cell that specifically binds with a cognate stimulatory ligand present on an antigen presenting cell.

A "stimulatory ligand," as used herein, means a ligand that when present on an antigen presenting cell (e.g., an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a "stimulatory molecule") on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands are well-known in the art and encompass, inter cilia, an MHC Class I molecule loaded with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

With respect to the cytoplasmic domain, the CAR molecule of some embodiments of the invention can be designed to comprise the CD28 and/or 4-1BB signaling domain by itself or be combined with any other desired cytoplasmic domain(s) useful in the context of the CAR molecule of some embodiments of the invention. In one embodiment, the cytoplasmic domain of the CAR can be designed to further comprise the signaling domain of CD3-zeta. For example, the cytoplasmic domain of the CAR can include but is not limited to CD3-zeta, 4-1BB and CD28 signaling modules and combinations thereof.

According to some embodiments of the invention, the intracellular domain comprises at least one, e.g., at least two, at least three, at least four, at least five, e.g., at least six of the polypeptides selected from the group consisting of: CD3 ζ (CD247, CD3z), CD28, 41BB, ICOS, OX40, and CD137.

According to some embodiments of the invention, the intracellular domain comprises the CD3 ζ -chain [CD247 molecule, also known as "CD3-ZETA" and "CD3z"; GenBank Accession NOs. NP_000725.1 and NP_932170.1], which is the primary transmitter of signals from endogenous TCRs.

According to some embodiments of the invention, the intracellular domain comprises various co-stimulatory protein receptors to the cytoplasmic tail of the CAR to provide additional signals to the T cell (second generation CAR). Examples include, but are not limited to, CD28 [e.g., GenBank Accession Nos. NP_001230006.1, NP_001230007.1, NP_006130.1], 4-1BB [tumor necrosis factor receptor superfamily, member 9 (TNFRSF9), also known as "CD137", e.g., GenBank Accession No. NP_001552.2], and ICOS [inducible T-cell co-stimulator, e.g., GenBank Accession No.

NP_036224.1]. Preclinical studies have indicated that the second generation of CAR designs improves the antitumor activity of T cells.

According to some embodiments of the invention, the intracellular domain comprises multiple signaling domains, such as CD3z-CD28-41BB or CD3z-CD28-
 5 OX40, to further augment potency. The term “OX40” refers to the tumor necrosis factor receptor superfamily, member 4 (TNFRSF4), e.g., GenBank Accession No. NP_003318.1 (“third-generation” CARs).

According to some embodiments of the invention, the intracellular domain comprises CD28-CD3z, CD3z, CD28-CD137-CD3z. The term “CD137” refers to tumor
 10 necrosis factor receptor superfamily, member 9 (TNFRSF9), e.g., GenBank Accession No. NP_001552.2.

According to some embodiments of the invention, when the CAR molecule is designed for a natural killer cell, then the signaling domain can be CD28 and/or CD3ζ. The transmembrane domain may be derived either from a natural or from a synthetic
 15 source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this invention may be derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137,
 20 CD154. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably
 25 between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

According to some embodiments of the invention, the transmembrane domain comprised in the CAR molecule of some embodiments of the invention is a transmembrane domain that is naturally associated with one of the domains in the CAR.

30 According to some embodiments of the invention, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the

transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

According to some embodiments, between the extracellular domain and the transmembrane domain of the CAR molecule, or between the cytoplasmic domain and the transmembrane domain of the CAR molecule, there may be incorporated a spacer domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular domain or, the cytoplasmic domain in the polypeptide chain. A spacer domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids.

According to an aspect of some embodiments of the invention, there is provided a method of treating cancer in a subject in need thereof, comprising administering to the subject the affinity binding entity, thereby treating the cancer in the subject.

Also provided is a use of the affinity binding entity as defined herein in the manufacture of a medicament for treating a pathology e.g., cancer.

The selection of the TCRL will naturally depend on its presentation in the pathology. Exemplary TCRLs and their association with pathologies are provided in the Tables hereinabove.

The term "treating" refers to inhibiting, preventing or arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein, the term "subject" includes mammals, preferably human beings at any age which suffer from the pathology.

The antibodies of some embodiments of the invention can be administered to an organism per se, or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as

physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the antibody accountable for the biological effect.

5 Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

10 Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

15 Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including
20 intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS)
25 include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion protein that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB;
30 pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the

infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide). However, each of these strategies has limitations, such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems, potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is disrupted, which renders it a suboptimal delivery method.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

The term “tissue” refers to part of an organism consisting of cells designed to perform a function or functions. Examples include, but are not limited to, brain tissue, retina, skin tissue, hepatic tissue, pancreatic tissue, bone, cartilage, connective tissue, blood tissue, muscle tissue, cardiac tissue brain tissue, vascular tissue, renal tissue, pulmonary tissue, gonadal tissue, hematopoietic tissue.

Pharmaceutical compositions of some embodiments of the invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with some embodiments of the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank’s solution, Ringer’s solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to some embodiments of the invention are conveniently delivered in the form of an

aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of some embodiments of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (TCRL-antibody) effective to

prevent, alleviate or ameliorate symptoms of a disorder (e.g., cancer) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

5 For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

10 Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of
15 administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide TCRL (the TCRL tissue) levels of the active ingredient are sufficient to induce or suppress the
20 biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated,
25 dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration,
30 the judgment of the prescribing physician, etc.

Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit (diagnostic or

therapeutic), which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice
5 associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions
10 comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

It is expected that during the life of a patent maturing from this application many relevant TCRLs will be developed and the scope of the term TCRLs is intended to
15 include all such new technologies *a priori*.

As used herein the term "about" refers to $\pm 10\%$.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

20 The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references
25 unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format
30 is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as

individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies
5 regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein
10 interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals there between.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known
15 manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term “treating” includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of
20 clinical or aesthetical symptoms of a condition.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base
25 deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

30 It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for

brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless
5 the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

10 EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and
15 recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John
20 Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed.
25 (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980);
30 available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074;

NO: 230 peptide or relevant control peptides (listed in the Table 15 below). Peptide loading efficiency was verified by using the ratio between MFI of HLA-A2-binding antibody BB7.2 on peptide-loaded T2 cells and MFI of unloaded T2 cells (>1) data not shown.

5 T2 or primary cells or cell lines (10^6) were incubated with 10 $\mu\text{g/ml}$ of specific Ab (with or without biotinylation) for 1 h at 4 °C, followed by incubation with PE-labeled anti-mouse/human/streptavidin Ab for 45 min at 4 °C. It will be appreciated that the work with anti mouse secondary antibody or with streptavidin gave similar results for D11 and B47B6. Cells were finally washed and analyzed by:

10 FACS 1:

Machine: BD FACS calibur

Analysis software: CELLQuest

FACS 2:

Machine: Beckman Coulter NAVIOS

15 Analysis software: Kaluza version 1.3

Production of TCR-like antibodies to HLA-A2/tyrosinase_{369–377}/ WT₁₂₆₋₁₃₄/MAGE-A₄₂₃₀₋₂₃₉/MAGE-A₉₂₂₃₋₂₃₁/PAP₁₁₂₋₁₂₀ using the hybridoma technique

HHD mice were immunized by 5-6 injections of HLA-A2-peptide complex 50 $\mu\text{g/mouse}$. 2-3 first injections were administered s.c with addition of QuilA adjuvant.

20 Hybridoma clones were generated by fusion of splenocytes isolated from mice immunized with the above complex (as previously described e.g., Weidanz et al. 2011 Int. Rev. Immunol. 30:328-340) with NSO myeloma cells and were screened and isolated by differential ELISA assays as described below. For example, for Tyrosinase TCRLs selection the relevant Tyr_{D369–377} peptide HLA-A2 complexes were used and compared to the non relevant p68-DDX5 control peptide (SEQ ID NO: 2 YLLPAIVHI) HLA-A2 complexes. ELISA with purified HLA-A2-Tyr complexes as well as with control HLA-A2 complex displaying other HLA-A2-restricted peptide (Table 15) was used to select specific clones. Isolated hybridoma clones were sub-cloned and were sequenced. Two clones 906-11-D11 (termed D11, Figure 68) and 905-2-D7 (termed D7,
30 Figure 69) were characterized.

Hybridomas were grown to >80% confluency in HAT DMEM or serum free DCCM2 medium and supernatant was collected. Purified IgG was isolated from culture

supernatant by affinity chromatography using Protein A column. SDS-PAGE analysis of the purified protein revealed homogenous, pure IgG with the expected molecular mass of ~150 kDa.

Construction of whole IgG Ab

5 The H and L Fab genes (only for MC1) were cloned for expression as human IgG1 κ Ab into the eukaryotic expression vectors the eukaryotic expression vectors pOptiVEC and pcDNA3.3-TOPO respectively. Each shuttle expression vector carries a different gene selection (for pOptiVEC the DHFR/HT- and for pcDNA3.3 Geneticin). Expression was facilitated by co-transfection of the two constructs into the
10 dihydrofolate reductase (DHFR)-deficient, Chinese hamster ovary (CHO)-derived DG44 cells in suspension culture by using the FreeStyle MAX reagent (Invitrogen). After co-transfection, cells were grown on selective medium. Clones that reacted specifically with JY T2 cells pulsed with tyrosinase 369–377 peptide were adapted to growth in 0.5 % serum and were further purified using protein A affinity
15 chromatography. SDS-PAGE analysis of the purified protein revealed homogenous, pure IgG with the expected molecular mass of ~150 kDa.

ELISA with supernatant or purified Abs

The binding specificities of individual supernatant or purified TCRL antibodies were determined by ELISA using biotinylated scMHC-peptide complexes. Maxi sorp
20 96 wells ELISA plates (Nunc #442404) were coated overnight with BSA-biotin (1 μ g/well). After having been washed, the plates were incubated (1 h, RT) with streptavidin (1 μ g/well), washed extensively, and further incubated (1 h, RT) with 0.25 μ g of MHC/peptide complexes. The plates were blocked for 30 min at RT with PBS/2% skim milk and subsequently were incubated for 1 h at RT with 1 μ g/well
25 supernatant or purified TCRL antibodies. After having been washed, the plates were incubated with HRP-conjugated/anti-human or mouse Ab. Detection was performed using TMB tetramethylbenzidine reagent (DAKO, S1599). The HLA-A2-restricted peptides used for specificity studies of the purified supernatant or purified TCRL antibodies.

Proteon XPR36 surface plasmon resonance (SPR) binding analysis

Immobilization of IgG TCR-like antibody was performed on a GLM (General Layer Medium) chip (Bio-Rad Laboratories, Hercules, CA, USA) at 25°C in the

vertical orientation and the continuous running buffer was PBST (10 mM Na-phosphate, 150 mM NaCl, and 0.005% Tween 20, pH 7.4). Five channels were activated with 50 μ l of a mixture of 0.04 M N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.01 M sulfo-N-hydroxysuccinimide (Sulfo-NHS) at a flow rate of 30 μ l/min. The anti-mouse or human IgG/NeutrAvidin was diluted in 10 mM sodium acetate buffer pH 4.5 to a final concentration of 25 μ g/ml and 150 μ l were injected followed by an injection of 150 μ l of 1 M ethanolamine-HCl pH 8.5. The IgG TCRL antibody/ purified biotinylated single-chain recombinant HLA-A2/Tyrosinase/WT1/MAGE-A4/MAGE-A9/PAP complex ligand was diluted in PBST to 5-10 μ g/ml and 90 μ l were injected in the vertical orientation with a flow rate of 30 μ l/min. The sixth channel remained empty to serve as a reference. The analyte purified single-chain recombinant HLA-A2/Tyrosinase/WT1/MAGE-A4/MAGE-A9/PAP complex/Fab TCRL antibody was injected (75 μ l at 50 μ l/min) in the horizontal orientation of the ProteOn using five different concentrations (1000, 500, 250, 125 and 62.5 nM). Running buffer was injected simultaneously in the sixth channel for double referencing to correct for loss of the captured antibodies from the chip sensor surface during the experiment. All binding sensorgrams were collected, processed and analyzed using the integrated ProteOn Manager (Bio-Rad Laboratories, Hercules, USA) software. Binding curves were fitted using the Langmuir model describing 1:1 binding stoichiometry, or with the Langmuir and mass transfer limitation model.

Functional assays

LDH-release assay

Bispecific TCRL redirected target cell killing was measured in a non-radioactive cytotoxicity assay using CytoTox96® (Promega). This assay quantitatively measures lactate dehydrogenase (LDH), an enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 10 minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color produced is proportional to the number of lysed cells.

Specifically, target cells and effector cells were washed, counted and resuspended in cRPMI medium (1% FBS) without phenol red. Target cells were adjusted to a cell density of 2.5×10^5 cells per ml and the effector cells at a cell density of 2.5×10^6 cells per ml. 40 μ l (1×10^4 cells) of target cells were cultured in a 96-well

V-shaped plate. A 5 times concentrated stock of the Bispecific TCRL test reagent was prepared at the highest test concentration, which was serially diluted 1 in 10 in medium without phenol red in a separate plate to obtain other test concentrations. The Bispecific TCRL was then added to the target cells in the assay plate at 20 µl per well to give the final indicated titrated amounts. The assay plate containing the target cells mixed with the Bispecific TCRL was then incubated for 20 minutes at 37 °C/5 % CO₂. Following the incubation, 40 µl effector cells (1 x 10⁵ cells) were added to each well resulting in an effector to target (E:T) ratio of 10:1. Control wells were set up with effector cells alone to calculate effector spontaneous release, target cells alone to calculate target spontaneous release, and target cells with 80 µg/ml digitonin final to calculate maximum release. Each condition was assayed in triplicates in a final volume of 100 µl. The plate was incubated at 37 °C/5 % CO₂ for 24 hours. Following the incubation period, the plate was centrifuged at 700 x g for 5 minutes and 50 µl transferred from each well to the corresponding well in a 96-well flat bottomed Maxisorb plate (Nunc). The CytoTox96® substrate mix was reconstituted using CytoTox96® assay buffer, as per manufacturer's instructions, and 50 µl added to each well of the plate. The plate was covered with aluminum foil and incubated at room temperature for 10 minutes. Then absorbance recorded at 490 nm on a plate reader. Percentage cytotoxicity was then calculated using the following equation: Specific lysis = [(Experimental – Effector Spontaneous – Target Spontaneous)/(Target Maximum – Target Spontaneous)] x 100. PBMCs for killing assays are isolated from healthy volunteers and with all regulatory IRBs approvals and written consents. Effector PBMCs are isolated using the Lymphoprep procedure.

Tumor cell lines and normal primary cells

Cells lines A375 (melanoma), U2OS (osteosarcoma), TCCSUP (bladder carcinoma) and Fib (fibroblasts) were cultured in complete DMEM supplemented with 10 % FBS (all supplied by GIBCO). 501A, SKMel5, Mewo and 1938 (melanoma), Saos2 (osteosarcoma), Panc1 (pancreatic carcinoma), J82 and UMUC3 (bladder), H1703 (non-small cell lung adenocarcinoma), JVM2 (Mantle cell lymphoma), IM9 (multiple myeloma), U266 (myeloma) and SW620 (colorectal adenocarcinoma) were cultured in complete RPMI supplemented with 10 % FBS (all supplied by GIBCO). Malme3m (melanoma), JEKO1 (mantle-cell lymphoma), SET2 (essential

thrombocythemia) and BV173 (B cell precursor leukemia) were cultures in complete RPMI supplemented with 20 % FBS (all supplied by GIBCO). THP-1 (AML) were cultured in complete RPMI supplemented with 10 % FBS (all supplied by GIBCO) and 0.05mM beta-mercaptoethanol (supplied by Thermo-fisher). OVCAR-3 (ovary adenocarcinoma) were cultured in complete RPMI supplemented with 20 % FBS (all supplied by GIBCO) and 0.01 mg/ml bovine insulin (supplied by Sigma). All cell lines were maintained at 37 °C in a humidified atmosphere of 7.5 % CO₂ and were purchased from American Type Culture Collection.

Normal primary hepatocytes, cardiac myocytes, osteoblasts, astrocytes, bronchial epithelial cells, colonic smooth muscle cells, urothelial cells and renal epithelial cells were obtained from Sciencell and cultured according to the manufacturer's instructions. All cell lines were maintained at 37 °C in a humidified atmosphere of 7.5 % CO₂.

Expression and purification of soluble recombinant Fab Abs in Expi293 system

The VH-CH1 and VL-CL genes of Tyr D11 and D7, MAGE-A4 C106B9, WT1 B47B6 and ESK1 IgGs were cloned for expression as Fab in the eukaryotic expression vector pcDNA3.4. His-tag was connected to the C-terminus of the CH1 region.

Expression was facilitated by co transfection of the two constructs (heavy and light chains) into the Expi293F human cells in Expi293 expression medium (both are components of the Expi293 expression system) by the Fectamine transfection reagent (Life technologies). Following co-transfection, cells were grown for 6 days. After 6 days cells were centrifuged at 700 X g for 5 minutes. Following centrifugation, the supernatant containing the D11, D7, C106B9, B47B6 or ESK1 Fab was removed from cells and filtered through 0.22μ filter. The supernatant was then dialyzed overnight against PBS.

The D11, D7, C106B9, B47B6 or ESK1 Fab recombinant protein was purified by metal affinity column (Talon) and dialyzed overnight against PBS. The purified D11, D7, C106B9, B47B6 or ESK1 Fab were analyzed on reduced and non-reduced SDS-PAGE.

Construction, Expression and purification of Bispecific TCRLs in Expi293 system

The VH-CH1 and VL-CL genes of Tyr D11 and D7, WT1 B47B6 and ESK1 and MAGE-A4 C106B9, IgGs were cloned for expression as bispecific (BS) in the eukaryotic expression vector pcDNA3.4 (sequences are shown in Figures 68-70, sequences of ESK1 is available from WO 2015/070061). For the light chain vector of Tyr D11, WT1 B47B6 and ESK1 and MAGE-A4 C106B9, anti CD3 (clone UCHT1) scFv was connected to the N-terminus of the VL region (BS format 3, #F3). For the heavy chain vector, His-tag was connected to the C-terminus of the CH1 region. For Tyr D7, anti CD3 (clone UCHT1) scFv was connected to the N-terminus of the VH region of the heavy chain (BS format 1, #F1) and His-tag was connected to the C-terminus of the CH1 region.

Expression was facilitated by co transfection of the two constructs into the Expi293F human cells in Expi293 expression medium (both are components of the Expi293 expression system) by the Fectamine transfection reagent (Life technologies). After co-transfection, cells were grown for 6 days. Following 6 days cells were centrifuged at 700 X g for 5 minutes. Following centrifugation, the supernatant containing the TCRL bispecific antibodies were removed from cells and filtered through 0.22 μ m filter. The supernatant was then dialyzed overnight against PBS.

The BS-TCRLs recombinant proteins were purified by two steps of metal affinity (Talon) and size exclusion chromatography (Superdex 200 10/300 GL GE). The purified BS-TCRLs were analyzed on SDS-PAGE.

In vivo assays

For 501A melanoma cell line (ATCC, Manassas VA, USA)

Cells were cultured in RPMI1640 growth medium (GIBCO, Waltham MA, USA) supplemented with 10 % fetal bovine serum (GIBCO, Waltham MA, USA). Human peripheral blood mononuclear cells (PBMC) were prepared from healthy donors by using SepMateTM-50 tubes (Stemcell).

At day 0, eight to ten weeks old female NOD/SCID mice (Envigo, Israel; n=6-8) were inoculated subcutaneously (s.c.) in a single flank with 5×10^6 501A melanoma cells +/- 25×10^6 PBMCs (Effector:Tumor cell ratio 5:1) in a final volume of 0.25 ml phosphate-buffered saline (PBS); D7 bispecific TCRL (0.1mg/kg) or vehicle (PBS)

were administered i.v. one hour after the s.c. inoculation in a final volume of 0.2 ml, with 4 additional doses administered every 24 hours.

For A375 melanoma cell line (ATCC, Manassas VA, USA)

Cells were cultured in RPMI1640 growth medium (GIBCO, Waltham MA, USA) supplemented with 10% fetal bovine serum (GIBCO, Waltham MA, USA).

Activated CD8 T-cells were prepared from human peripheral blood mononuclear cells (PBMC) using a rapid expansion protocol (REP). Naïve PBMCs were produced from healthy donor's peripheral blood using SepMate™-50 tubes (Stemcell), following CD8 T cells enrichment using Dynabeads® Untouched™ Human CD8 T Cells kit (Invitrogen). Activation of the purified CD8 T cells was performed in flasks pre-coated with monoclonal antibodies against CD3 (OKT3) and CD28 for 72 hrs in media supplemented with 10% FBS and 100 IU/mL of human IL-2. Activated cells were expanded over the period of 14 days in media supplemented with 10% FBS, 3000 IU/ml IL-2, 30ng/ml OKT3 and 2×10^8 irradiated PBMCs.

At day 0, eight to ten weeks old female NOD/SCID mice (Envigo, Israel; n=6-8) were inoculated subcutaneously (s.c.) in a single flank with 5×10^6 A375 melanoma cells +/- 10×10^6 REP CD8 T-cells (Effector: Tumor cell ratio 2:1) in a final volume of 0.25 ml phosphate-buffered saline (PBS); MAGE-A4 C106B9 bispecific TCRL (0.1mg/kg), WT1 B47B6 bispecific TCRL (0.1mg/kg) or vehicle (PBS) were administered i.v. one hour after the s.c. inoculation in a final volume of 0.2ml, with 4 additional doses administered every 24 hours.

In both cases (501A and A375) tumors were measured two times per week with calipers in two perpendicular dimensions and tumor volumes were calculated with the following formula: $width \times \left(\frac{length}{2}\right)^2 \times 3.14$

Other TCRL antibodies used in the present study

The generation of MC1 is described in WO2008/120202.

The generation of ESK1 (Dao T, Yan S, Veomett N, Pankov D, Zhou L, Korontsvit T, Scott A, Whitten J, Maslak P, Casey E, Tan T, Liu H, Zakhaleva V, Curcio M, Doubrovina E, O'Reilly RJ, Liu C, Scheinberg DA. Targeting the intracellular WT1 oncogene product with a therapeutic human antibody. Sci Transl Med. 2013 Mar 13;5(176):176ra33). ESK1 was thus generated by synthetic gene synthesis according to the published sequence WO 2015/070061 ESK1 full VH - SEQ ID NO:128 and ESK1

full VL - SEQ ID NO: 130 in the sequence listing of WO 2015/070061. The antibody was produced in HEK293 cells as IgG using the Expi293 system as described above and was purified from culture supernatants using protein A affinity chromatography.

Extraction of nucleic acids

5 Total RNA was extracted from 1×10^6 - 5×10^6 cells cultured cells with RNeasy Plus Mini (Qiagen) according to the manufacturer's instructions.

cDNA synthesis

cDNA was synthesized from 1-5 μ g RNA, using a combination of oligo dT and random hexamer (1:1) with SuperScript® III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. For quantitative PCR, cDNA was diluted 10 1:5 with H₂O.

Conventional PCR (PCR)

The PCR cycling conditions were 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 20 s, 60 °C for 1 min and 72 °C for 1 min. The PCR was ended with a final extension of 72 °C for 10 min. Reactions were performed with KAPA HiFi PCR Kit 15 (Kapa Biosystems) according to the manufacturer's instructions.

Following primers were used:

TYR_S: TTAGCAAAGCATACCATCA (SEQ ID NO: 3) and TYR_AS: CCAGACAAAGAGGTCATAA (SEQ ID NO: 4)

20 for tyrosinase expression (expected product size: 117bp) and WT1_S: AGGCTGCAATAAGAGATA (SEQ ID NO: 5) and WT1_AS: TTCGCTGACAAGTTTTAC (SEQ ID NO: 6) for WT1 expression (expected product size: 188bp).

To visualize the amplified products, 10 μ L of samples were mixed with 2 μ L of 25 6x loading buffer (New England Biolabs) and subjected to electrophoresis on 1.5 % agarose gels stained with ethidium bromide with DNA markers (New England Biolabs). The presence and intensity of the PCR product bands was determined on an ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Quantitative PCR (qPCR)

30 Quantitative PCR was carried out using TaqMan Gene Expression Master Mix on a ABI 7300 instrument (Applied Biosystems), according to the manufacturer's instructions. The cycle conditions for real-time PCR were 95°C for 10 min, followed by

40 cycles of 95°C for 15 sec, and 60°C for 1 min. Probes for real-time PCR were purchased from Applied Biosystems; at the 5' end, they were conjugated to the fluorochrome FAM. Following assays (primers and probes) were used: for TYR (cat# Hs00165976), for MAGE A4 (cat# Hs00751150), and for WT1 (cat# Hs01103751).

- 5 Beta-actin was used as a housekeeping gene for normalization (cat# Hs999999903).

Peptides used in the present study

<i>Table 4 - Ala Scan - TyrD</i>		
Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:
TyrD-A1	AMDGTMSQV	104
TyrD-A2	YADGTMSQV	105
TyrD-A3	YMAGTMSQV	106
TyrD-A4	YMDATMSQV	107
TyrD-A5	YMDGAMSQV	108
TyrD-A6	YMDGTASQV	109
TyrD-A7	YMDGTMAQV	110
TyrD-A8	YMDGTMSAV	111
TyrD-A9	YMDGTMSQA	112

<i>Table 5 - Similar peptides - TyrD</i>			
Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:	Similar to
Tyrosinase D (Tyrosinase peptide)	YMDGTMSQV	113	
Tyrosinase N	YMNGTMSQV	114	
*KIAA0355	YMDNVMSEV	115	TyrD
KPNA1	VMDSKIVQV	116	TyrD
GPLD1	LMNGTLKQV	117	TyrD
TyrD-S1	SQDGTRSQV	118	TyrD
TyrD-S2	VMDTTKSQV	119	TyrD
TyrD-S3	GMDGTQQQI	120	TyrD
TyrD-S4	GMVGTMTVEV	121	TyrD
TyrD-S5	MMDATFSAV	122	TyrD
TyrD-S6	QMDPTGSQL	123	TyrD

*TyrD-S7	SMDGSMRTV	124	TyrD
TyrD-S8	WMDGIASQI	125	TyrD
TyrD-S9	YLEGILSQV	126	TyrD
TyrD-S10	YMAIKMSQL	127	TyrD
TyrD-S11	YMDAVVSLV	128	TyrD
TyrD-S12	YMDGTNRRI	129	TyrD
TyrD-S13	YMDPSTYQV	130	TyrD
TyrD-S14	YMLGTNHQL	131	TyrD
TyrD-S15	YMPGTASLI	132	TyrD
TyrD-S16	YMRETRSQL	133	TyrD
*TyrD-S17	MMDGAMGYV	134	TyrD
*TyrD-S18	NMDSFMAQV	135	TyrD
*TyrD-S19	QMDFIMSCV	136	TyrD
*TyrD-S20	YEDLKMYQV	137	TyrD
*TyrD-S21	YMDTIMELV	138	TyrD
*TyrD-S22	YTDLAMSTV	139	TyrD
*TyrD-S23	YVDFVMSSV	140	TyrD

* Ala-based similar peptides

<i>Table 6- Similar peptides - WT1</i>			
Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:	Similar to
WT1 (WT1 peptide)	RMFPNAPYL	141	
WT1-S1	LDFPNLPYL	142	WT1
*WT1-S2	RCFPNCPFL	143	WT1
WT1-S3	LMFENAAYL	144	WT1
WT1-S4	RMFPNKYSL	145	WT1
WT1-S5	RLFPNAKFL	146	WT1
*WT1-S6	RLFPNLPEL	147	WT1
*WT1-S7	RMFPTPPSL	148	WT1
WT1-S8	RMVPRAVYL	149	WT1
WT1-S9	RMFFNGRYI	150	WT1
WT1-S10	RMLPHAPGV	151	WT1
WT1-S11	YMFPNAPYL	152	WT1
WT1-S12	AMDPNAAYV	153	WT1

WT1-S13	ICFPNAPKV	154	WT1
WT1-S14	NMFENGCYL	155	WT1
WT1-S15	NMPPNFPYI	156	WT1
WT1-S16	REMTQAPYL	157	WT1
WT1-S17	RMAPRAPWI	158	WT1
WT1-S18	RMEPRAPWI	159	WT1
WT1-S19	RMEPRAPWV	160	WT1
WT1-S20	RMFLNNPSI	161	WT1
WT1-S21	RMFQQTFYL	162	WT1
WT1-S22	RMNPNPSI	163	WT1
WT1-S23	RQFPNASLI	164	WT1
WT1-S24	RQFPNKDAL	165	WT1
WT1-S25	RVFPWASSL	166	WT1
WT1-S26	RLFPWGNKL	167	WT1

* Ala-based similar peptides

Table 7 - Ala Scan – WT1		
Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:
WT1-A1	AMFPNAPYL	168
WT1-A2	RAFPNAPYL	169
WT1-A3	RMAPNAPYL	170
WT1-A4	RMFANAPYL	171
WT1-A5	RMFPAAPYL	172
WT1-A7	RMFPNAAYL	173
WT1-A8	RMFPNAPAL	174
WT1-A9	RMFPNAPYA	175

Table 8 - Similar peptides – MAGE-A4			
Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:	Similar to
MAGE-A4 (MAGE-A4 peptide)	GVYDGREHTV	176	
MAGE-A4-S1	GLADGRTHTV	177	MAGE-A4
MAGE-A4-S2	GVSDGRWHSV	178	MAGE-A4
MAGE-A4-S4	GVYDGEEHSV	179	MAGE-A4

MAGE-A4-S5	GLYDGMEHL	180	MAGE-A4
MAGE-A4-S6	GVSDGQWHTV	181	MAGE-A4
MAGE-A4-S9	GVYAGREHFL	182	MAGE-A4
MAGE-A4-S10	GLYDGMEHLI	183	MAGE-A4
MAGE-A4-S12	ASYDGTEVTV	184	MAGE-A4
MAGE-A4-S13	AVLDGRELRV	185	MAGE-A4
MAGE-A4-S15	GLYDGIEHFM	186	MAGE-A4
MAGE-A4-S16	GLYDGPVHEV	187	MAGE-A4
MAGE-A4-S17	GVCAGREHFI	188	MAGE-A4
MAGE-A4-S18	GVYAGRPLSV	189	MAGE-A4
MAGE-A4-S19	TVYDLREQSV	190	MAGE-A4
MAGE-A4-S20	VVDDGVEHTI	191	MAGE-A4
MAGE-A4-S21	GVFDGLHTV	192	MAGE-A4

Table 9 - Ala Scan – MAGE-A4

Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:
MAGE-A4-A1	AVYDGREHTV	193
MAGE-A4-A2	GAYDGREHTV	194
MAGE-A4-A3	GVADGREHTV	195
MAGE-A4-A4	GVYAGREHTV	196
MAGE-A4-A5	GVYDAREHTV	197
MAGE-A4-A6	GVYDGAETV	198
MAGE-A4-A7	GVYDGRAHTV	199
MAGE-A4-A8	GVYDGREATV	200
MAGE-A4-A9	GVYDGREHAV	201
MAGE-A4-A10	GVYDGREHTA	202

Table 10 - Similar peptides – MAGE-A9

Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:	Similar to
MAGE-A9 (MAGE-A9 peptide)	ALSVMGVYV	203	
MAGE-A9S1	ALSVLGVMV	204	MAGE-A9
MAGE-A9S3	ALSRKGIYV	205	MAGE-A9
MAGE-A9S4	ALSVMYSYL	206	MAGE-A9

MAGE-A9S6	AVSHMGVLV	207	MAGE-A9
MAGE-A9S7	LLSLMGVLV	208	MAGE-A9
*MAGE-A9S8	VLSIMGVYA	209	MAGE-A9
MAGE-A9S10	ALQVRKVYV	210	MAGE-A9
MAGE-A9S11	ALQVYGVEV	211	MAGE-A9
MAGE-A9S13	ALSVAGGFV	212	MAGE-A9
MAGE-A9S14	ALSVLGKVV	213	MAGE-A9
MAGE-A9S15	ALSVMIPAV	214	MAGE-A9
MAGE-A9S16	DLSVCSVYV	215	MAGE-A9
MAGE-A9S17	ILGVMGVDV	216	MAGE-A9
MAGE-A9S20	LLSVNGVSV	217	MAGE-A9
MAGE-A9S23	SLSPMGRYV	218	MAGE-A9
MAGE-A9S24	ALSAVMGVTL	219	MAGE-A9
MAGE-A9S25	AILLVMGVDV	220	MAGE-A9
MAGE-A9S26	ALSDHHVYL	221	MAGE-A9

* Ala-based similar peptides

Table 11 - Ala Scan – MAGE-A9

Peptide name	Peptide-HLA-A2 sequence/	SEQ ID NO:
MAGE-A9-A2	AASVMGVYV	222
MAGE-A9-A3	ALAVMGVYV	223
MAGE-A9-A4	ALSAMGVYV	224
MAGE-A9-A5	ALSVAGVYV	225
MAGE-A9-A6	ALSVMGVYV	226
MAGE-A9-A7	ALSVMGAYV	227
MAGE-A9-A8	ALSVMGVAV	228
MAGE-A9-A9	ALSVMGVYA	229

Table 12 - Similar peptides – PAP

Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:	Similar to
PAP (PAP peptide)	TLMSAMTNL	230	
PAP(TLM)S1	TLMSAEANL	231	PAP
PAP(TLM)S2	QLCSAMTQL	232	PAP
PAP(TLM)S3	RLMSALTQL	233	PAP

PAP(TLM)S4	GLMSLTNNL	234	PAP
PAP(TLM)S5	GLMSMATNL	235	PAP
PAP(TLM)S6	GLMSMTNNL	236	PAP
PAP(TLM)S7	LLMSISTNL	237	PAP
PAP(TLM)S8	QLPSTMTNL	238	PAP
PAP(TLM)S9	TLASSMGNL	239	PAP
PAP(TLM)S10	TLFSALTGL	240	PAP
PAP(TLM)S11	TLGSATTEL	241	PAP
PAP(TLM)S12	TLMRAMTDC	242	PAP
PAP(TLM)S13	TLMSMVANL	243	PAP
PAP(TLM)S14	TLPSAETAL	244	PAP
PAP(TLM)S15	TLPSRMTVL	245	PAP
PAP(TLM)S18	RLMSALTQV	246	PAP
PAP(TLM)S19	SIHSQMTNL	247	PAP
PAP(TLM)S20	SIMFAMTPL	248	PAP
PAP(TLM)S21	TIVAAMSNNL	249	PAP
PAP(TLM)S22	TLITAMEQL	250	PAP
PAP(TLM)S23	TLTSNMSQL	251	PAP

Table 13 - Ala Scan – PAP

Peptide name	Peptide-HLA-A2 sequence	SEQ ID NO:
PAP A1	ALMSAMTNNL	252
PAP A3	TLASAMTNNL	253
PAP A4	TLMAAMTNNL	254
PAP A6	TLMSAATNNL	255
PAP A7	TLMSAMANL	256
PAP A8	TLMSAMTAL	257
PAP A9	TLMSAMTNA	258

Table 14- Similar peptides found in normal essential tissues by MS.

Peptide name	Peptide sequence/SEQ ID NO:	Gene	Normal tissue in which peptide was found by MS
KPNA1	VMDSKIVQV/259	KPNA1,KPNA5,KPNA6	Adrenal, bladder, brain cerebellum, brain cerebral

			cortex, brain cerebrum, colon, heart, intestine, kidney, liver, lung, mesothelial, nerve, pituitary, retina, spinal cord cervical, adipose, breast, duodenum, esophagus, gallbladder, ovary, pancreas, prostate, skin, spleen, stomach, testis, uterus
WT1-S10	RMLPHAPGV/260	HDAC1,HDAC2	Adrenal, bladder, brain cerebellum, brain cerebral cortex, brain cerebrum, colon, heart, intestine, kidney, liver, lung, mesothelial, nerve, pituitary, retina, spinal cord cervical, adipose, breast, duodenum, esophagus, gallbladder, ovary, pancreas, prostate, skin, spleen, stomach, testis, uterus
WT1-S12	AMDPNAAYV/261	SERPINA6	Liver
WT1-S22	RMNPNSPSI/262	ERH	Colon, intestine, kidney, lung, duodenum, gallbladder, uterus
MAGE-A4-S1	GLADGRTHTV/263	THBS3	Colon, endothelium, intestine, kidney, mesothelial, nerve, pituitary, duodenum, stomach
MAGE-A4-S16	GLYDGPVHEV/264	DPYSL4	Brain cerebellum, brain cerebrum, intestine, lung, prostate, spleen
MAGE-A4-S21	GVFDGLHTV/265	BTD	Brain cerebral cortex, intestine, kidney, liver, lung, mesothelial, retina, breast, duodenum, stomach, testis, uterus
MAGE-A9-S26	ALSDHHVYL/266	ALDOC	Adrenal, bladder, brain cerebellum, brain cerebral cortex, brain cerebrum, colon, endothelium, heart, intestine, kidney, liver, lung, mesothelial, nerve, pituitary, retina, spinal cord cervical, breast, duodenum, esophagus, prostate skin, spleen, stomach, testis, uterus
PAP-S3	RLMSALTQL/267	DAB2IP	Brain cerebellum, brain cerebral cortex, brain cerebrum, colon, heart, intestine, kidney, lung, mesothelial, nerve, retina, spinal cord cervical, adipose, breast, duodenum, prostate, spleen, uterus
PAP-S18	RLMSALTQV/268	RASAL2	Bladder, brain cerebellum,

			brain cerebral cortex, brain cerebrum, colon, endothelium, heart, intestine, kidney, liver, lung, mesothelial, nerve, pituitary, retina, spinal cord cervical, adipose, breast, duodenum, esophagus, gallbladder, ovary, prostate, skin, spleen, stomach, testis, uterus
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<i>Table 15 - Control peptides</i>		
Peptide	Peptide-HLA-A2 sequence	SEQ ID NO:
MART1(26)	ELAGIGILTV	269
CMV	NLVPMVATV	270
Gag	SLYNTVATL	271
Tyrosinase D	YMDGTMSQV	272
WT-1	RMFPNAPYL	273
MAGE-A4	GVYDGREHTV	274
PAP	TLMSAMTNL	275
MAGE-A9	ALSVMGVYV	276
SSX-2	KASEKIFYV	277
NY-ESO	SLLMWITQC	278
UHRF1	TLFDYEVRL	279

5

EXAMPLE I:**TCR-LIKE ANTIBODIES FOR HLA-A2/Tyrosinase*****Isolation of Abs with TCR-like specificity to HLA-A2/tyrosinase₃₆₉₋₃₇₇***

Generation of MHC-TyrD₃₆₉₋₃₇₇ complex - Previous studies performed by the present inventors have shown the generation of recombinant antibodies with peptide-specific, HLA-A2-restricted specificity to tumor and viral T cell epitopes using large antibody phage libraries. These molecules are termed TCR-like antibodies. To generate antibodies with a specificity to the HLA-A2/TyrD₃₆₉₋₃₇₇ complex, recombinant peptide-HLA-A2 complexes were generated that present the Tyrosinase peptide (tyrosinase₃₆₉₋₃₇₇ YMDGTMSQV, SEQ ID NO: 1) using a single chain MHC construct.

15 HHD mice were immunized by 5-6 injections of HLA-A2-peptide complex 50

µg/mouse. 2-3 first injections were administrated s.c with addition of QuilA adjuvant. Hybridoma clones were generated by fusion of splenocytes isolated from immunized mice (as previously described e.g., Weidanz et al. 2011 Int. Rev. Immunol. 30:328-340) with NSO myeloma cells and were screened and isolated by differential ELISA assays as described above using TyrD369–377 peptide and HLA-A2 complexes folded with p68-DDX5 control peptide. ELISA with purified HLA-A2-Tyr complexes as well as with control HLA-A2 complex displaying other HLA-A2-restricted peptide was used to select specific clones. Isolated hybridoma clones were sub-cloned and were sequenced. Two clones 906-11-D11 (termed D11, Figure 69) and 905-2-D7 (termed D7, Figure 68) were characterized.

Characterization of TCR-like antibodies with specificity to HLA A2/tyrosinase 369–377

To determine the apparent affinity of isolated TCR-like antibodies, surface plasmon resonance (SPR) binding analysis was used in which the isolated purified IgG TCR-like antibody was immobilized to the SPR sensor chip by using anti-mouse IgG to indirectly immobilize the TCR-like antibodies on the chip surface. The analyte is the purified single-chain recombinant HLA-A2/Tyrosinase complex used at various concentrations. As shown in Figure 1, the sensorgrams of SPR analysis revealed similar affinity for the HLA-A2/Tyrosinase specific TCR-like antibody clones MC1, D11, and D7 with corresponding affinity of 4.1 nM for MC1 and D11 and 3.8 nM for D7. These results indicate that all three TCR-like antibody clones exhibited similar high affinity of 4nM towards the specific HLA-A2/peptide complex.

To investigate the fine peptide epitope specificity of the isolated TCR-like antibodies towards the Tyrosinase 369-377 peptide alanine scanning was performed in which specific residues in the peptide were mutated to alanine and the binding of the TCR-like antibodies to Ala mutated peptides was tested by their loading onto T2 antigen presenting cells. Binding was monitored by flow cytometry and extent of binding of TCR-like antibodies to the mutated presented peptides as measured by mean fluorescence intensity (MFI) was compared in comparison to T2 APCs loaded with the native unmutated Tyrosinase peptide. The proper loading of the various Ala mutated peptides (described in Figure 2) was monitored by flow cytometry using BB7.2 a monoclonal antibody for HLA-A2.

All Ala mutated peptides were efficiently loaded onto T2 cells in comparison to the native un-mutated Tyrosinase peptide (data not shown). Peptide loading efficiency is verified using the ratio between MFI of HLA-A2-binding Ab BB7.2 on peptide-loaded T2 cells and MFI of unloaded T2 cells (>1). As shown in Figure 2, all three TCR-like antibodies exhibited peptide dependency binding as specific mutations affected the binding and induced a decrease in the binding intensity of the TCR-like antibody upon introduction of Ala at specific peptide positions. These results indicate that all three TCR-like antibodies exhibited peptide-specific and restricted binding in the context of HLA-A2 loaded with various Ala mutated Tyrosinase peptides, indicating that these antibodies are TCR-like in their binding properties, thus, they bind the MHC-peptide complex with MHC-restricted and peptide-specific manner.

However, the three TCR-like antibodies differ in their fine specificity and peptide-dependent reactivity with the number of positions in the peptide that were sensitive to Ala mutation and affected binding sensitivity. As MC1 exhibited a marked decrease of 90% in binding to a single Ala mutated peptide at one position # 6, D11 and D7 exhibited a decrease of $>90\%$ at two positions # 3, 6 for D11 and a decrease of $>90\%$ for D7 binding at four positions # 3, 4, 6, 7. A milder but highly significant decrease of $>70\%$ in three positions # 1, 3, 6 was further observed for MC1 binding to Ala mutated peptides while D11 and D7 exhibited significant decrease in binding of $>70\%$ when 5 peptide residues were mutated to Ala (positions # 1, 2, 3, 4, 6 for D11 and positions # 2, 3, 4, 6, 7 for D7).

Overall, the Alanine scanning analysis reveals that D11 and D7 are more influenced and sensitive to Ala mutations compared to MC1 as observed by the ability of the various Ala mutated Tyr peptide to bind properly the Tyr specific TCR-like antibodies. According to the data presented in Figure 2, D11 and D7 are more peptide restricted and sensitive in their binding properties compared to MC1; they are sensitive (not including anchor positions) to Ala mutations in 4 out of 9 peptide residues while MC1 only to 3 positions. D11 and D7 are even sensitive in their binding properties in a 5th position 7, and 5, respectively. Specifically, D11 decrease the binding in 68% at position #7, 67% position #5, 59% position #8; D7 decrease the binding in 66% at position #5, 63% position #1, 63% position #8.

It is concluded that Ala scanning can be used as a measure to determine the selectivity and fine specificity of TCR-like antibodies. As more sensitivity to Ala mutations is exhibited the more specific and peptide-dependent binding will be observed. This strategy can be used to filter and select for the optimal TCR-like antibodies that exhibited the higher and optimized selectivity and specificity properties as MHC-restricted peptide-specific binders.

Binding selectivity and specificity of TCR-like antibodies towards HLA-A2/Tyrosinase

To characterize the binding specificity of the isolated TCR-like antibodies the reactivity and specificity of the purified IgGs were assessed by flow cytometry. T2 APCs were loaded with specific or control peptides and incubated with the Ab, followed by incubation with PE-labeled anti-human or mouse. Ab. As shown in Figures 3-7, the MC1 (Figure 7), D11, and D7 (Figures 3-6) IgGs bound T2 cells loaded with the tyrosinase peptide but did not bind significantly to cells loaded with control peptides (Table 15). Very low background binding was observed on control peptides with MFIs ratio of 3-7 for MC1 (Figure 7) while D11 and D7 did not exhibit any background binding (Figure 3-6). The extent of loaded peptide presentation was monitored by binding of MAbs BB7.2 which binds all HLA-A2 peptide complexes. These results indicate that all three TCR-like antibodies exhibited HLA-A2-restricted peptide-specific binding as they bound only to cells presenting the Tyrosinase but no other HLA-A2 restricted peptides.

To explore whether the HLA-A2/tyrosinase TCR-like Abs are capable of binding endogenously derived MHC-tyrosinase complexes on the surface of tumor cells, flow cytometry analysis was done on lines derived from melanoma patients. Cells were incubated with anti-tyrosinase 369–377/HLA-A2 TCR-like antibodies Ab followed by incubation with PE-labeled anti-human or anti-mouse Ab. As shown in Figures 8-12 the TCR-like antibodies recognized tyrosinase-positive and HLA-A2-positive cells with a very high intensity. As shown this indicates that large numbers of HLA-A2-tyrosinase complexes are presented on the surface of the melanoma cells. The staining with the TCR-like antibodies was very homogeneous; intracellular staining of these melanoma cells (for example 624.38, and 501A) with Ab against the tyrosinase protein revealed that ~95% of the cells in each line tested express the tyrosinase protein

(data not shown). No reactivity was detected with tyrosinase-negative or HLA-A2-negative cells. The specificity of the anti-tyrosinase/HLA-A2 TCR-like Abs was verified by extensive flow cytometry analysis of multiple cell lines of various histological origins which are HLA-A2 positive and Ag (tyrosinase) negative. This analysis is shown in Figures 10-12. D11 and D7 reactivity was tested also on a panel of normal primary cells including endothelial cells, fibroblasts, astrocytes, hepatocytes, renal cells, cardiac myocytes, colonic muscle, and PBMCs (Figures 13-17). No binding to these HLA-A2+ and Tyr- normal primary cells was observed while background binding was observed when MC1 was tested on PBMCs (Figure 17). Summary of the analysis of D11 and D7 reactivity with HLA-A2+/Tyrosinase+ melanoma cells as well as extensive panel of HLA-A2+/Tyrosinase- cells of various histological origins including the normal primary cells is presented in Figures 18-19. D11 and D7 TCR-like antibodies reactivity looks extremely specific only to melanoma cells expressing HLA-A2 and the antigen tyrosinase.

The overall conclusion from these studies is that the TCR-like Abs are specific and they recognize only the specific peptide-MHC complex presented on the cell surface when the adequate combination of HLA allele and Ag exist. However, careful evaluation of flow cytometry data exhibited results that demonstrate differential selectivity of MC1 compared to D11 and D7. For example, analysis of binding of MC1 to HLA-A2+ and Tyr- cell lines HepG2, SW620, and Loucy as shown in Figure 9 reveals background binding as measured by MFI, however, similar analysis of D11 and D7 on these cells revealed no binding (Figure 10 and 12). Side by side comparison of the three TCR-like antibodies on these and additional cells (Figure 12) revealed that MC1 exhibited significant binding to HLA-A2+/Tyr+ melanoma cells but had background binding on a variety of HLA-A2+/Tyr- cells (SW620, Colo205, HepG2, Panc1, RPMI, DG75, Jeko1, and Loucy) while D11 and D7 did not exhibit any background binding to these cells.

It may thus be concluded that D11 and D7 are more specific and selective compared to MC1 and that comprehensive flow cytometry studies as well as other assays, for example, functional assays utilizing a large panel of cells of different histological origins that express the appropriate HLA allele and are positive or negative for the antigen are useful tools to evaluate the selectivity of TCR-like antibodies.

To further evaluate the fine specificity of the Tyrosinase specific TCR-like antibodies their reactivity with peptides that exhibit sequence similarity to the native tyrosinase was evaluated (Table 5).

Thus, another round of similar peptides selection is performed when Alanine/Glycine scanning data are available as described above for a particular TCR-like antibody. Based on alanine scanning the contribution of each amino acid residue in the peptide antigen to TCRL binding is measured and evaluated. Similar peptides that preserve the critical positions are identified by the above described tools and are assigned higher priority. These peptides are synthesized and used for fine specificity evaluation as described above.

The strategy described here combines in silico analysis of peptide sequence similarity combined with Mass spectroscopy analysis of eluted HLA peptides, peptide data bases and alanine scanning provides a tool box to fully control peptide search parameters, more than other tools such as BLAST or ScanProsite provide. Additional parameters are employed including the range of allowed peptide lengths, the maximum allowed number or differences in sequence, and the requirement for HLA binding score. The tool also applies the ability to define certain amino acids as equivalent. Most important is the ability to highlight peptides that have been found by mass spectrometry or by peptide databases.

Applying the above tools, the fine specificity of the three TCR-like antibodies was evaluated by synthesizing a large panel of similar peptides that have been selected for evaluation according to the criteria described herein (Table 5). These similar peptides have been loaded on T2 APCs and the reactivity of the TCR-like antibodies was tested. As shown in Figure 20, when MC1 was tested on a panel of similar peptides in comparison with binding to native tyrosinase peptide it was observed that it exhibits background binding to peptides with sequence similarity to Tyrosinase such as KIAA0335 and KPNA1. However, as shown in Figures 21-28, the D11 and D7 TCR-like antibodies did not bind any similar peptide from a large panel of such that were analyzed by peptide loading including no recognition of the KIAA0335 and KPNA1 peptides that exhibited background binding with MC1. These data demonstrate the superior selectivity and fine specificity of D11 and D7 in comparison to MC1 and demonstrates the usefulness of the similar peptide approach and tools developed as

described above as important tools to evaluate the selectivity and fine specificity hierarchy when evaluating a panel of TCR-like antibodies for the best and optimal candidate for further evaluation.

Moreover, after alanine scanning of TCR-like antibodies additional similar peptides have been selected and tested. Since each amino acid within the TyrD peptide sequence is unlikely to contribute equally to Tyr TCRL binding, the peptide residues critical for recognition by the Tyr TCRL were identified. A set of synthetic peptides were produced in which each amino acid of the TyrD 9-mer was sequentially replaced by alanine. The ability of Tyr TCRL to bind cells pulsed with each of these alanine - substituted peptides was determined by FACS analysis and the binding results was compared to those obtained with the non-mutated peptide. The residue at position that alanine substitution result in a large decrease in binding compared to the non-mutated peptide, was considered critical. A directed in-silico search was then carried out to identify protein sequences that contain only the critical positions motif. These peptides were also utilized for specificity evaluation of Tyr TCRLs (Table 5 S17-S23). These alanine scanning analysis-derived similar peptides were synthesized and loaded onto T2 APCs cells and the reactivity of D11 and D7 was tested. As show in Figure 28, no binding to these peptides was observed, thereby further confirming and strengthening the fine specificity and selectivity of these TCR-like antibodies.

EXAMPLE IA

CHARACTERIZATION OF TCR-LIKE ANTIBODIES FOR HLA-A2/Tyrosinase

Comparison of the fine specificity of Abs with TCR-like specificity to HLA-A2/tyrosinase369–377

To characterize the binding specificity of the isolated TCR-like antibodies the reactivity and specificity of the purified IgGs (with or without biotinylation) were assessed by flow cytometry. T2 APCs were loaded with Tyrosinase peptide or control peptides (Table 15) and incubated with the Ab (D7, D11 or MC1), followed by incubation with PE-labeled streptavidin or PE-labeled anti mouse Abs. As shown in Figure 38, D11, and D7 TCRLs bound T2 cells loaded with the tyrosinase peptide but showed no binding to cells loaded with control peptides. In contrast, MC1 TCRL

showed binding to T2 cells loaded with both the Tyrosinase peptide and with the irrelevant peptide used as control.

To further evaluate the specificity of the D7 and D11 TCR-like antibodies their reactivity with peptides that exhibit sequence similarity to the tyrosinase peptide was evaluated. The peptides are shown in Table 5.

As shown in Figure 39 MC1 TCRL exhibits readily detectable binding to various peptides with sequence similarity to Tyrosinase peptide such as KIAA0335 and KPNA1 (Table 14) as well as to peptides marked as S2, S4, S5, S9, S11, S13, S18, (S19, S22 and S23). D11 and D7 TCR-like antibodies did not bind any of the peptides from this same panel of similar peptides. These data demonstrate the superior selectivity and fine specificity of D11 and D7 TCRLs compared to MC1 TCRL and demonstrates the usefulness of the similar peptide approach and tools developed as described above to evaluate the selectivity and fine specificity hierarchy of TCRLs.

The present inventors explored binding specificity of the HLA-A2/tyrosinase TCR-like Abs to MHC-tyrosinase peptide complexes endogenously displayed on the surface of melanoma cell lines. Cells were incubated with anti-tyrosinase 369–377/HLA-A2 TCR-like antibodies Ab (with or without biotinylation) followed by incubation with PE-labeled streptavidin or anti-mouse Abs. A panel of tumor cells and normal primary cells that have been characterized for HLA-A2 (positive) and Tyrosinase (positive or negative) expression was used to compare the binding of the TCR-like antibodies. As shown in Figure 40A-C, the TCR-like antibodies recognized tyrosinase-positive and HLA-A2-positive cells. The TCR-Like antibodies were tested on multiple HLA-A2-positive cell lines of various origin that do not show Tyr RNA expression (Tyr-negative). As shown in Figures 40A-B, D11 and D7 TCRLs did not bind any of these cells while MC1 readily stained various HLA-A2+/Tyr- cells. D7 and D11 TCRLs did not exhibit any binding to normal primary cells, while MC1 displayed detectable binding to some of them (Figure 40C).

Overall, D7 and D11 TCRLs demonstrated superior specificity and selectivity recognizing tyrosinase peptide presented by HLA-A2 compared to MC1 TCRL.

Functional assays were used to further characterize the D7 and D11 TCR-like antibodies. TCRLs variable regions were fused to an anti-CD3 scFv which can re-target effector T cells to kill tumor target cell in a of bi-specific format. As shown in Figures

41-44, D7 and D11 CD3 Bi-specific TCR-like antibody constructs showed robust cytotoxicity against melanoma 501A cells in vitro in the presence of human PBMCs. Panc-1, Tyrosinase negative cell line served as negative control and demonstrated no cytotoxicity. No cytotoxicity was detected against a panel of HLA-A2+/Tyr- normal human primary cells with D7 and D11 TCRLs confirming their selectivity.

EXAMPLE IB

In vivo efficacy of D7 BS TCRL in s.c. 501A melanoma tumor formation model in NOD/SCID mice

Figure 45 shows in vivo efficacy of D7 BS TCRL in S.C. 501A melanoma tumor formation model in NOD/SCID mice. Clearly, administration of the bispecific antibody completely inhibited tumor formation over 65 days of the experiment, as evidenced by tumor volume. The results support the use of variable sequences of the TCRLs described herein in clinical settings.

EXAMPLE II

TCR-LIKE ANTIBODIES FOR HLA-A2/WT1

Isolation and characterization of Abs with TCR-like specificity to HLA-A2/WT1

To generate such antibodies with a specificity to the HLA-A2/WT1 complex, recombinant peptide-HLA-A2 complexes were generated that present the WT1 peptide (RMFPNAPYL, SEQ ID NO: 151) using a single chain MHC construct. The generation of antibodies was as described in the general materials and methods as well as in Example I above, A TCR-like specific clone termed B47 (also referred to as B47B6) was isolated and characterized (Figure 70).

As a comparison for TCR-like antibody binding selectivity, a TCR-like antibody termed ESK1 Dao T, Yan S, Veomett N, Pankov D, Zhou L, Korontsvit T, Scott A, Whitten J, Maslak P, Casey E, Tan T, Liu H, Zakhaleva V, Curcio M, Doubrovina E, O'Reilly RJ, Liu C, Scheinberg DA.

The binding affinity of B47 was evaluated by surface plasmon resonance (SPR) binding analysis in which the isolated purified IgG TCR-like antibody was immobilized to the SPR sensor chip by using anti-mouse IgG to indirectly immobilize the TCR-like

antibodies on the chip surface. The analyte is the purified single-chain recombinant HLA-A2/WT1 complex used at various concentrations. As shown in Figure 29, the sensorgrams of SPR analysis revealed an affinity for the HLA-A2/WT1 specific TCR-like antibody clone B47 of 4.4nM.

To characterize the binding specificity of the isolated TCR-like antibodies the reactivity and specificity of the purified IgGs were assessed by flow cytometry. T2 APCs were loaded with specific or control peptides (Table 15) and incubated with the Ab, followed by incubation with PE-labeled anti-human or mouse Ab. As shown in Figures 30 and 31, B47 and ESK1 bound T2 cells loaded with the WT1 peptide (Figure 30) but did not bind to cells loaded with control peptides (Figure 31). Of significance difference was the binding intensity observed for B47 and ESK1. While B47 bound intensely to T2 cells loaded with 10^{-4} - 10^{-5} M peptide, ESK1 bound much weaker to T2 cells loaded with 10^{-4} M WT1 peptide (MFI 18 for ESK1 compared with 474 for B47). At peptide concentration of 10^{-5} M B47 still bound significantly (MFI 88) while binding of ESK1 was almost undetectable or very low (Figure 30). These results indicated marked differences in the affinity and binding sensitivity of B47 compared to ESK1 with sharp decrease in the binding intensity of ESK1 compared to B47 with 10 x decreases in peptide concentration. B47 and ESK1 did not bind T2 APCs loaded with control HLA-A2 restricted peptides (Figure 31). These results indicate that both TCR-like antibodies exhibited HLA-A2-restricted peptide-specific binding as they bound only to cells presenting the WT1 but no other HLA-A2 restricted peptides.

To further investigate the WT1 TCR-like antibodies fine specificity evaluation of binding to similar peptides identified in silico with the strategy described above was performed. As shown in Figures 32 and 33, B47 did not bind any similar peptide from a designed panel (Table 6). However, as shown in Figure 32, ESK1 exhibited low background binding with two similar peptides. B47 was evaluated on additional control peptides and similar peptides (Figure 34). Further analysis of these TCR-like antibodies was performed by flow cytometry using tumor cells that are HLA-A2 and express or not the WT1 antigen. As shown in Figure 35, the ESK1 WT1 TCR-like antibody bound intensely to HLA-A2+/WT+ BV173 and SET2 cells however B47 did not exhibit any binding to these cells to the level of flow cytometry sensitivity. To further investigate specificity the reactivity of ESK1 and B47 was evaluated on cells that are HLA-A2 but

do not express the WT1 gene as evaluated by PCR. As shown B47 did not bind to any of these cells while ESK1 bound to 501, A498, and SKMEL cells that were found to be WT1 negative. Other WT1 negative cells were not bound by ESK1. The level of HLA-A2 expression was monitored with MAb BB7.2 which recognizes all HLA-A2/peptide molecules on the cell surface. A summary of binding data for B47 WT-specific TCR-like antibody is shown in Figure 36.

To further investigate the conflicting data of the binding of ESK1 and B47 to HLA-A2+/WT1+ BV173 and SET2 cells, i.e binding could be detected significantly by ESK1 but not B47 we employed direct biochemical means to evaluate actual WT1 presentation on these cells. We employed HLA peptide elution strategies from various tissues as well from BV173 and SET2 cells followed by MS analysis of eluted peptides. The data of these experiments indicate that the WT1 peptide has not been detected in any of the MS runs of clinical tissues or cell lines. In depth analysis of the BV173 or SET-2 cell lines (mRNA WT1-positive) failed to detect the peptide (Orbitrap or Q Exactive MS instruments). The WT1 peptide was detected by OrbiTrap MS following direct elution from T2 peptide-loaded cells. These T2 cells were loaded with various WT1 peptide concentrations of 10^{-5} , 10^{-7} , 10^{-9} M and the peptide was detected by the MS in elutions from T2 APCs loaded with peptide concentration of 10^{-5} and 10^{-7} M. Detecting the peptide from T2 cells loaded at 10^{-7} M peptide by the MS corresponds to actual presentation of ~250 sites/cell (using the Orbitrap MS).

These data exemplifies the usefulness of the described binding tools towards peptide loaded cells that display similar peptides and cells of various histological origins to evaluate the specificity and selectivity of TCR-like antibodies.

To further investigate epitope specificity, alanine scanning mutagenesis was performed on the WT1 peptide sequence. As shown in Figure 37 which demonstrates that only mutation in position 1 of the WT1 peptide influenced the binding intensity of ESK1 indicating that the binding selectivity and fine specificity of ESK1 is limited compared to B47 as also observed for the specificity pattern as observed for similar peptides and for cells that are HLA-A2+/WT1-/. These data suggest that the selectivity and fine specificity of B47 is superior compared to ESK1 and that the tool box presented herein is a valuable tool to evaluate the selectivity and fine specificity of

TCR-like antibodies in the process of their selection, characterization, and pre-clinical development.

EXAMPLE IIA

TCR-LIKE ANTIBODIES FOR HLA-A2/WT1

Comparison of fine specificity of Abs with TCR-like specificity to HLA-A2/WT1

The selectivity of TCR-like antibodies B47 and ESK1 both recognizing WT1 peptide was compared (Dao et al. Sci Transl Med. 2013 Mar 13;5(176):176ra33)

T2 APCs were loaded with specific (WT1, SEQ ID NO: 141) or control peptides (Table 15) and incubated with the B47 and ESK1 antibodies, followed by incubation with PE-labeled streptavidin or anti- mouse Abs. Both B47 and ESK1 TCRLs bound T2 cells loaded with the WT1 peptide but did not bind to cells loaded with control peptides (Figure 46). A panel of similar peptides (Table 6) was synthesized to further characterize specificity of the WT1 TCRLs. The B47 TCRL did not bind to any of the similar peptides loaded onto T2 cells while ESK1 showed detectable binding to several similar peptides (Figure 47). ESK1 TCRL showed binding to a similar peptide derived from HDAC2 (Histone deacetylase 2, Table 14) that is ubiquitously presented by many normal cells. WT1-S10 (SEQ ID NO: 151) is presented in normal tissues as evidenced by mass spectrometry in brain, cerebral cortex, heart, kidney, liver, lung, and other normal tissues (Table 14).

Further characterization of binding of B47 and ESK1 TCRLs by SPR showed that affinity of B47 (5 nM) is much stronger than that of ESK1 (200 nM) mainly due to faster dissociation rate of ESK1 and MHC-WT1 peptide complexes (Figure 48). Additional alanine scanning mutagenesis of the WT1 peptide was performed to refine peptide epitope specificity of B47 TCR-like antibodies (Figure 49). The mutant peptides were loaded onto T2 cells and binding assay was performed as described above. The loading of the various Ala mutants was monitored by flow cytometry using BB7.2 monoclonal antibody against HLA-A2.

As shown in Figure 49, substitutions to Ala at some positions significantly affected B47 binding to the mutated peptides. B47 TCRL exhibited greater sensitivity to positional substitutions (as compared to ESK1, Figure 37). The B47 TCR-like antibody lost >73% of its binding to presented peptide with when 4 residues in the peptide were mutated to Alanine (positions 1, 3, 4, and 7). A 5th position sensitivity can be attributed

to position number 5. For both B47 and ESK1 TCRLs position 2 was critical as it is expected to serve as anchor position for the peptides in the HLA-A2 peptide binding groove.

Further characterization and comparison between B47 and ESK1 TCRLs was done on tumor cell lines and primary cells of various origins. As shown in Figure 50, B47 did not bind to a panel of cells that were all HLA-A2 positive and WT1 mRNA positive or negative cells. In contrast, ESK1 TCRL bound to a number of both tumor and normal primary cells (all HLA-A2+). For example, JVM2 and IM9 (both HLA-A2 positive and WT1 negative) as well as normal primary astrocytes showed binding. Cytotoxicity assays using TCRL-aCD3 bi-specific constructs and human PBMCs showed that B47 TCRL did not induce death of HLA-A2+/WT1+ or HLA-A2+/WT1- cells while ESK1 TCRL-aCD3 was cytotoxic to a number of cells, including WT-1 negative. Thus, B47 TCRL demonstrate superior specificity in both binding and functional activity in the bi-specific format compared to ESK1 that binds to and re-targets CD3 T-cells toward some cells, including normal primary cells, regardless of WT-1 expression.

EXAMPLE III

TCR-Like Antibodies with specificity to HLA-A2/MAGE-A4

EXAMPLE IIIA

Isolation and characterization of TCRL with specificity to HLA-A2/MAGE-A4

To characterize the binding specificity of the isolated TCR-like antibodies the reactivity and specificity of the purified IgGs were assessed by flow cytometry. T2 APCs were loaded with MAGE-A4 peptide or control peptides (Table 15) and incubated with the TCRL Ab C106B, followed by incubation with PE-labeled streptavidin or PE-labeled anti mouse Abs. As shown in Figure 52, C106B9 bound T2 cells loaded with the MAGE-A4 peptide but showed no binding to cells loaded with control peptides.

To further evaluate the specificity of the C106B9 TCR-like antibody its reactivity with peptides that exhibit sequence similarity to the MAGE-A4 peptide was evaluated.

The peptides are shown in Table 8.

As shown in Figure 53, C106B9 TCRL did not bind any of the peptides from this panel of similar peptides. These data demonstrate the high selectivity and fine

specificity of C106B9 and demonstrates the usefulness of the similar peptide approach and tools developed as described above to evaluate the selectivity and fine specificity of TCRLs.

To determine the apparent affinity of the isolated TCR-like antibody, surface plasmon resonance (SPR) binding analysis was used in which the isolated purified IgG TCR-like antibody was immobilized to the SPR sensor chip by using anti-mouse IgG to indirectly immobilize the TCR-like antibodies on the chip surface. The analyte is the purified single-chain recombinant HLA-A2/MAGE-A4 complex used at various concentrations. As shown in Figure 54, the sensorgrams of SPR analysis revealed affinity for the HLA-A2/MAGE-A4 specific TCR-like antibody clone C106B9 with corresponding affinity of 8.8nM.

To investigate the fine peptide epitope specificity of the isolated TCR-like antibodies towards the MAGE-A4 peptide alanine scanning was performed in which specific residues in the peptide were mutated to alanine and the binding of the TCR-like antibody to Ala mutated peptides was tested by their loading onto T2 antigen presenting cells (Table 9). Binding was monitored by flow cytometry and extent of binding of TCR-like antibodies to the mutated presented peptides as measured by mean fluorescence intensity (MFI) was compared in comparison to T2 APCs loaded with the native unmutated MAGE-A4 peptide. The proper loading of the various Ala mutated peptides (described in Figure 2) was monitored by flow cytometry using BB7.2 a monoclonal antibody for HLA-A2.

All Ala mutated peptides were efficiently loaded onto T2 cells in comparison to the native un-mutated MAGE-A4 peptide (data not shown). As shown in Figure 55, The TCR-like antibody exhibited peptide dependent binding as specific mutations affected the binding and induced a decrease in the binding intensity of the TCR-like antibody upon introduction of Ala at specific peptide positions. These results indicate that MAGE-A4 TCR-like antibody exhibited peptide-specific and restricted binding in the context of HLA-A2 loaded with various Ala mutated MAGE-A4 peptides, indicating that this antibody is TCR-like in its binding properties, thus, it binds the MHC-peptide complex with MHC-restricted and peptide-specific manner.

The C106B9 TCR-like antibody exhibited a marked decrease of 90 % in binding to Ala mutated peptide at four positions # 4, 5, 6, and 7. A 5th position sensitivity can be attributed to position number 2 (decrease of 33%).

Overall, the Alanine scanning analysis reveals that Ala scanning can be used as a measure to determine the selectivity and fine specificity of TCR-like antibodies. As more sensitivity to Ala mutations is exhibited the more specific and peptide-dependent binding will be observed. This strategy can be used to filter and select for the optimal TCR-like antibodies that exhibited the higher and optimized selectivity and specificity properties as MHC-restricted peptide-specific binders.

The present inventors explored binding specificity of the HLA-A2/MAGE-A4 TCR-like Ab to MHC- MAGE-A4 peptide complexes endogenously displayed on the surface of tumor cell lines. Cells were incubated with anti-MAGE-A4- HLA-A2 TCR-like antibodies Ab followed by incubation with PE-labeled streptavidin or anti-mouse Abs. A panel of tumor cells and normal primary cells that have been characterized for HLA-A2 (positive) and MAGE-A4 (positive or negative) expression was used to compare the binding of the TCR-like antibodies. As shown in Figure 56, the TCR-like antibody recognized with low intensity MAGE4-positive and HLA-A2-positive cells. The TCR-Like antibodies were tested on multiple HLA-A2-positive cell lines of various origin that do not show MAGE-A4 RNA expression (MAGE-A4-negative), killing activity of these cells with a MAGE-A4/HLA-A2 TCRL-Bispecific construct was also tested. As shown in Figures 56, C106B9 TCRL did not bind any of these cells.

Functional assays were used to further characterize the C106B9 TCR-like antibody. TCRLs variable regions were fused to an anti-CD3 scFv which can re-target effector T cells to kill tumor target cell in a of bi-specific format. As shown in Figures 57, the C106B9 Bi-specific TCR-like antibody constructs showed robust cytotoxicity against MAGE-A4 positive cells in vitro in the presence of human PBMCs. TCCSUP and OVCAR, MAGE-A4 negative cell line served as negative control and demonstrated no cytotoxicity. As further shown in Figure 58, No cytotoxicity was detected against a panel of HLA-A2+/MAGE-A4- normal human primary cells with C106B9 TCRL confirming its selectivity.

EXAMPLE IIIB

In vivo efficacy of MAGE-A4 C106B9 BS TCRL in s.c. A375 melanoma tumor formation model in NOD/SCID mice

Figure 59 shows in vivo efficacy of C106B9 BS TCRL in S.C. A375 melanoma tumor formation model in NOD/SCID mice. Clearly, administration of the bispecific antibody completely inhibited tumor formation over 35 days of the experiment, as evidenced by tumor volume. The results support the use of variable sequences of the TCRLs described herein in clinical settings.

EXAMPLE IV

TCR-Like Antibodies with specificity to HLA-A2/MAGE-A9

Isolation and characterization of TCRL with specificity to HLA-A2/MAGE-A9

To characterize the binding specificity of the isolated TCR-like antibodies the reactivity and specificity of the purified IgGs were assessed by flow cytometry. T2 APCs were loaded with MAGE-A9 peptide or control peptides and incubated with the TCRL Ab F184C7, followed by incubation with PE-labeled streptavidin or PE-labeled anti mouse Abs. As shown in Figure 60, F184C7 bound T2 cells loaded with the MAGE-A9 peptide but showed no binding to cells loaded with control peptides.

To further evaluate the specificity of the F184C7 TCR-like antibody its reactivity with peptides that exhibit sequence similarity to the MAGE-A9 peptide was evaluated. The peptides are shown in Table 10.

As shown in Figure 61, F184C7 TCRL did not bind any of the peptides from this panel of similar peptides. These data demonstrate the high selectivity and fine specificity of F184C7 and demonstrates the usefulness of the similar peptide approach and tools developed as described above to evaluate the selectivity and fine specificity of TCRLs.

To investigate the fine peptide epitope specificity of the isolated TCR-like antibodies towards the MAGE-A9 peptide alanine scanning was performed in which specific residues in the peptide were mutated to alanine and the binding of the TCR-like antibody to Ala mutated peptides was tested by their loading onto T2 antigen presenting cells (Table 11). Binding was monitored by flow cytometry and extent of binding of TCR-like antibodies to the mutated presented peptides as measured by mean

fluorescence intensity (MFI) was compared in comparison to T2 APCs loaded with the native unmutated MAGE-A9 peptide. The proper loading of the various Ala mutated peptides (described in Figure 2) was monitored by flow cytometry using BB7.2 a monoclonal antibody for HLA-A2.

5 All Ala mutated peptides were efficiently loaded onto T2 cells in comparison to the native un-mutated MAGE-A9 peptide (data not shown). As shown in Figure 62, The TCR-like antibody exhibited peptide dependency binding as specific mutations affected the binding and induced a decrease in the binding intensity of the TCR-like antibody upon introduction of Ala at specific peptide positions. These results indicate
10 that MAGE-A9 TCR-like antibody exhibited peptide-specific and restricted binding in the context of HLA-A2 loaded with various Ala mutated MAGE-A9 peptides, indicating that this antibody is TCR-like in its binding properties, thus, it binds the MHC-peptide complex with MHC-restricted and peptide-specific manner.

The F184C7 TCR-like antibody exhibited a marked decrease of 90 % in binding
15 to five Ala mutated peptide at five positions # 3, 5, 6, 7 and 8.

Overall, the Alanine scanning analysis reveals that Ala scanning can be used as a measure to determine the selectivity and fine specificity of TCR-like antibodies. As more sensitivity to Ala mutations is exhibited the more specific and peptide-dependent binding will be observed. This strategy can be used to filter and select for the optimal
20 TCR-like antibodies that exhibited the higher and optimized selectivity and specificity properties as MHC-restricted peptide-specific binders.

The present inventors explored binding specificity of the HLA-A2/MAGE-A9 TCR-like Ab to a panel of normal primary cells of various origin that do not show MAGE-A9 RNA expression. As shown in Figures 63, F184C7 TCRL did not bind any of these
25 cells. Positive control was T2 cells loaded with the MAGE-A9 peptide to which F184C7 bound intensely.

EXAMPLE V

TCR-Like Antibodies with specificity to HLA-A2/PAP

30 Isolation and characterization of TCRL with specificity to HLA-A2/PAP

To characterize the binding specificity of the isolated TCR-like antibodies the reactivity and specificity of the purified IgGs were assessed by flow cytometry. T2

APCs were loaded with PAP peptide or control peptides and incubated with the TCRL Ab D10A3, followed by incubation with PE-labeled streptavidin or PE-labeled anti mouse Abs. As shown in Figure 64, D10A3 bound T2 cells loaded with the PAP peptide but showed no binding to cells loaded with control peptides.

5 To further evaluate the specificity of the D10A3 TCR-like antibody its reactivity with peptides that exhibit sequence similarity to the PAP peptide was evaluated. The peptides are shown in Table 12.

As shown in Figure 65, D10A3 TCRL did not bind any of the peptides from this panel of similar peptides. These data demonstrate the high selectivity and fine
10 specificity of D10A3 and demonstrates the usefulness of the similar peptide approach and tools developed as described above to evaluate the selectivity and fine specificity of TCRLs.

To investigate the fine peptide epitope specificity of the isolated TCR-like antibodies towards the PAP peptide alanine scanning was performed in which specific
15 residues in the peptide were mutated to alanine and the binding of the TCR-like antibody to Ala mutated peptides was tested by their loading onto T2 antigen presenting cells (Table 13). Binding was monitored by flow cytometry and extent of binding of TCR-like antibodies to the mutated presented peptides as measured by mean fluorescence intensity (MFI) was compared in comparison to T2 APCs loaded with the
20 native unmutated PAP peptide. The proper loading of the various Ala mutated peptides (described in Figure 2) was monitored by flow cytometry using BB7.2 a monoclonal antibody for HLA-A2.

All Ala mutated peptides were efficiently loaded onto T2 cells in comparison to the native un-mutated PAP peptide (data not shown). As shown in Figure 66, The TCR-
25 like antibody exhibited peptide dependency binding as specific mutations affected the binding and induced a decrease in the binding intensity of the TCR-like antibody upon introduction of Ala at specific peptide positions. These results indicate that PAP TCR-like antibody exhibited peptide-specific and restricted binding in the context of HLA-A2 loaded with various Ala mutated PAP peptides, indicating that this antibody is TCR-
30 like in its binding properties, thus, it binds the MHC-peptide complex with MHC-restricted and peptide-specific manner.

The D10A3 TCR-like antibody exhibited a marked decrease of 90% in binding to three Ala mutated peptide at three positions # 3, 6, and 8. Decrease of 70% in binding to one Ala mutated peptide at position # 4 was also observed. A 5th position sensitivity can be attributed to position number 7 (decrease of 45%).

5 The present inventors explored binding specificity of the HLA-A2/PAP TCR-like Ab to a panel of normal primary cells of various origin that do not show PAP RNA expression. As shown in Figures 67, D10A3 TCRL did not bind any of these cells. Positive control was T2 cells loaded with the PAP peptide to which D10A3 TCRL bound strongly.

10 Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

15 All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission
20 that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. An affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 Heavy		
Chain (HC)	SEQ ID NO: 309	SYGVH
CDR2 HC	SEQ ID NO: 310	VIWAGGTTNYSALMS
CDR3 HC	SEQ ID NO: 311	DGHFHFDF
the CDR1 Light		
Chain (LC)	SEQ ID NO: 303	RASDIIYSNLA
CDR2 LC	SEQ ID NO: 304	AATNLAA
CDR3 LC	SEQ ID NO: 305	QHFWGSSIS

said affinity binding entity capable of binding HLA-A2/Tyr_{D369-377} in an MHC restricted manner.

2. An affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 Heavy Chain		
(HC)	SEQ ID NO: 293	TSGMGVS
CDR2 HC	SEQ ID NO: 294	HIYWDDDKRYNPSLKS
CDR3 HC	SEQ ID NO: 295	KDYGSSFYAMHY
the the CDR1 Light		
Chain (LC)	SEQ ID NO: 287	KASQDIHNYIA
CDR2 LC	SEQ ID NO: 288	YTSTLQP
CDR3 LC	SEQ ID NO: 289	LQYDNLWT

said affinity binding entity capable of binding HLA-A2/Tyr_{D369-377} in an MHC restricted manner.

3. An affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 325	SYDMS
CDR2 HC	SEQ ID NO: 326	YMSSGGGTYPDTVKG
CDR3 HC	SEQ ID NO: 327	HDEITNFDY
CDR1 LC	SEQ ID NO: 319	RASQISNSLH
CDR2 LC	SEQ ID NO: 320	YASQIS
CDR3 LC	SEQ ID NO: 321	QQSYSWPLT

said affinity binding entity capable of binding HLA-A2/ WT₁₂₆₋₁₃₄ in an MHC restricted manner.

4. An affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 341	GYWIE
CDR2 HC	SEQ ID NO: 342	EILPGSGGTNYNEKFKG
CDR3 HC	SEQ ID NO: 343	DSNSFTY
CDR1 LC	SEQ ID NO: 335	SVSSSVDYIH
CDR2 LC	SEQ ID NO: 336	STSILAS
CDR3 LC	SEQ ID NO: 337	QQRSSYT

said affinity binding entity capable of binding HLA-A2/ MAGE-A4₃₂₈₋₃₄₃ in an MHC restricted manner.

5. An affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 357	FSSSWMN
CDR2 HC	SEQ ID NO: 358	RIYPGDGDNTNYNEKFKG
CDR3 HC	SEQ ID NO: 359	EATTVVAPYYFDY
CDR1 LC	SEQ ID NO: 351	RASENIYRNLA
CDR2 LC	SEQ ID NO: 352	AATNLAD
CDR3 LC	SEQ ID NO: 353	QHFWGTPLT

said affinity binding entity capable of binding HLA-A2/ MAGE-A9₃₄₄₋₃₅₉ in an MHC restricted manner.

6. An affinity binding entity comprising an antigen binding domain comprising CDR sequences which are N-C ordered:

CDR1 HC	SEQ ID NO: 373	DYNMD
CDR2 HC	SEQ ID NO: 374	DINPNYDTTTYNQKFKG
CDR3 HC	SEQ ID NO: 375	RNYGNYVGFDY
CDR1 LC	SEQ ID NO: 367	KASQRVNNDVA
CDR2 LC	SEQ ID NO: 368	YASNRYT
CDR3 LC	SEQ ID NO: 369	QQDYSSPFT

said affinity binding entity capable of binding HLA-A2/ PAP₃₆₀₋₃₇₅ in an MHC restricted manner.

7. The affinity binding entity of any one of claims 1-6, being selected from the group consisting of an antibody, a CAR and a TCR.

8. The affinity binding entity of any one of claims 1-6, being an antibody.

9. The affinity binding entity of any one of claims 1-6, being a TCR.
10. The affinity binding entity of any one of claims 1-6, being a CAR.
11. The affinity binding entity of any one of claims 1-8, being a soluble entity.
12. The affinity binding entity of any one of claims 7 or 8, being a humanized antibody.
13. The affinity binding entity of any one of claims 1-8, 11-12 comprising a therapeutic moiety.
14. The affinity binding entity of any one of claims 1-8, 11-12 comprising a therapeutic moiety.
15. The affinity binding entity of any one of claims 1-8, 11-12 comprising a detectable moiety.
16. The affinity binding entity of any one of claims 7-8, 11-15, wherein said antibody is a single chain antibody, a bi-specific antibody or a full length antibody.
17. An isolated polynucleotide comprising a nucleic acid sequence encoding the affinity binding entity of any one of claims 1-16.
18. An expression vector comprising the polynucleotide of claim 17 operably linked to a cis-acting regulatory element.
19. A cell comprising the polynucleotide of claim 17 or the expression vector of claim 18.

20. A pharmaceutical composition comprising the affinity binding entity of any of claims 1-16, the vector of claim 18 or the cell of claim 19.

21. A method of detecting a cancer cell, comprising contacting the cell with the antibody of any one of claims 7-8, 11-16, under conditions which allow immunocomplex formation, wherein a presence of said immunocomplex or level thereof is indicative of the cancer cell.

22. A method of diagnosing and treating cancer in a subject in need thereof, comprising:

- (a) detecting the presence of cancer cells in the subject according to the method of claim 21;
- (b) diagnosing the subject as having cancer when cancer cells are detected;
- (c) treating the subject with an anti-cancer therapy.

23. A method of diagnosing cancer in a subject in need thereof, comprising contacting a cell of the subject with the antibody of any one of claims 7-8, 11-16, under conditions which allow immunocomplex formation, wherein a presence of said immunocomplex or level thereof is indicative of the cancer.

24. The method of claim 22 or 23, wherein said cell is a skin cell.

25. A method of treating a cancer, comprising administering to a subject in need thereof a therapeutically effective amount of the affinity binding entity of any one of claims 1-16, the vector of claim 19 or the cell of claim 19, thereby treating the cancer.

26. Use of the affinity binding entity of any one of claims 1-16, the vector of claim 19 or the cell of claim 19 in the manufacture of a medicament for treating cancer.

27. The method of any one of claims 21-25 or use of claim 26, wherein when said affinity binding entity is for TyrD said cancer is selected from the group consisting of melanoma and glioblastoma.

28. The method of any one of claims 21-25 or use of claim 26, wherein when said affinity binding entity is for WT1 said cancer is selected from the group consisting of chronic myelocytic leukemia, multiple myeloma (MM), acute lymphoblastic leukemia (ALL), acute myeloid/myelogenous leukemia (AML), myelodysplastic syndrome (MDS), mesothelioma, ovarian cancer, gastrointestinal cancers e.g., colorectal cancer adenocarcinoma, thyroid cancer, breast cancer, lung cancer (e.g., non small cell lung cancer), melanoma, osteosarcoma, endometrial cancer, prostate cancer and glioblastoma.²² The method of any one of claims 21-25 or use of claim 26, wherein when said affinity binding entity is for MAGE-A4 said cancer is selected from the group consisting of melanoma, ovarian cancer, T cell leukemia/lymphoma (e.g., ATLL), testicular cancer, head and neck cancer, bladder cancer and esophagus cancer.

29. The method of any one of claims 21-25 or use of claim 26, wherein when said affinity binding entity is for MAGE-A9 said cancer is selected from the group consisting of renal cell carcinoma, bladder cancer, breast cancer and hepatocellular carcinoma.

30. The method of any one of claims 21-25 or use of claim 26, wherein when said affinity binding entity is for PAP said cancer is selected from the group consisting of prostate cancer.