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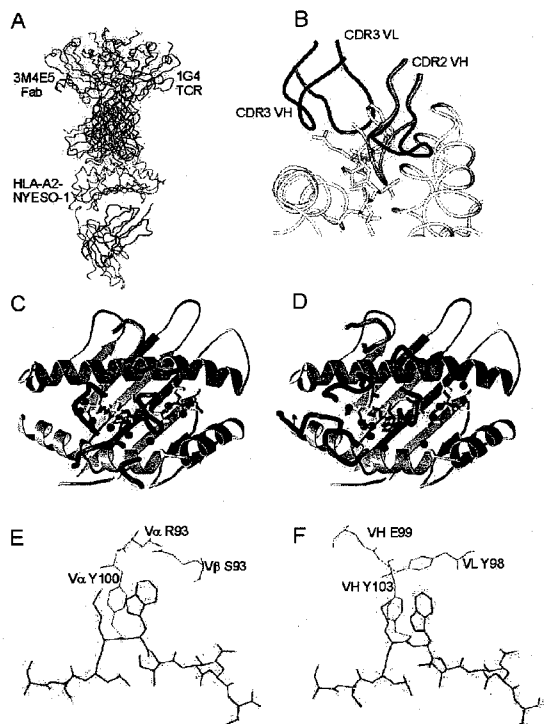
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[Continued on next page]

(54) Title: HIGH AFFINITY T-CELL RECEPTOR-LIKE NY-ESO-1 PEPTIDE ANTIBODIES, METHODS, AND USES THEREOF

FIGURE 1



(57) Abstract: The present invention relates to specific binding members, particularly antibodies and fragments thereof, which bind to NY-ESO-1 tumor antigen peptide/MHC molecule complexes, particularly in a manner like T cell receptors and with greater affinity than T cell receptors recognizing NY-ESO-I peptide/HLA complexes. These antibodies, particularly human antibodies are useful in the diagnosis and treatment of cancer, including melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma. The antibodies and fragments thereof of the present invention may also be used in therapy in combination with chemotherapeutics, immune modulators, or anti-cancer agents and/or with other antibodies or fragments thereof. Antibodies of this type are exemplified by the novel antibodies T1, T2 and T3 whose sequences are provided herein.



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HIGH AFFINITY T-CELL RECEPTOR-LIKE NY-ESO-1 PEPTIDE ANTIBODIES, METHODS, AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates to specific binding members, particularly antibodies and fragments thereof, which bind to NY-ESO-1 tumor antigen peptide/MHC molecule complexes, particularly in a manner like T cell receptors and with greater affinity than T cell receptors recognizing NY-ESO-1 peptide/HLA complexes. These antibodies, particularly human antibodies, are useful in the diagnosis and treatment of cancer, including melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma. The antibodies and fragments thereof of the present invention may also be used in therapy in combination with chemotherapeutics, immune modulators, or anti-cancer agents and/or with other antibodies or fragments thereof.

BACKGROUND OF THE INVENTION

[0002] NY-ESO-1 is a germ cell antigen that is aberrantly expressed by different tumor types (Gnjatic, S. et al (2006) Adv. Cancer Res 95:1-30). This antigen was originally discovered by screening a tumor-derived cDNA expression library with autologous serum of an esophageal cancer patient. Thus, NY-ESO-1 was discovered based on its capacity to induce an antibody response *in vivo* in cancer patients. This humoral response is restricted to cancer patients and not seen in healthy individuals (Stockert, E. et al (1998) J. Exp. Med. 187:1349-1354). The gene coding for NY-ESO-1, known as CTAG1, is a gene with expression limited to germ cells and is not expressed in normal somatic tissue. NY-ESO-1 is frequently expressed in cancer and normal testis, and is thus termed a cancer testis (CT) antigen (Scanlan, M.J. et al (2004) Cancer Immunol 4:9). The NY-ESO-1 gene maps to the Xq28 region of the X chromosome and codes for several products (Chen, Y.T. et al (1997) Proc Natl Acad Sci 94:1914-1918). The main product of NY-ESO-1 is a 180 amino acid long 18 KDa protein, with a glycine-rich N-terminal region and an extremely hydrophobic C-terminal region, which is very insoluble and can be confused for a transmembrane domain. The function of NY-ESO-1 remains unknown.

[0003] NY-ESO-1 monoclonal antibodies ES121, E978 and B9.8 have been developed and confirm its expression in early spermatagonia with loss of expression upon sperm cell differentiation (Jungbluth,

A.A. et al (2001) In J. Cancer 92:856-860; Schulz-Thater, E. et al. (2000) Br J. Cancer 83:204-208; Vaughan, H.A. et al (2004) Clin Cancer Res 10:8396-8404). NY-ESO-1 expression in primary or metastatic cancer tissue has been assessed by RT-PCR analysis, immunohistochemistry or Western blot. NY-ESO-1 is seen in approximately one-third to one-quarter of all melanoma, lung, esophageal, liver, gastric, prostate, ovarian and bladder cancers (Gnjatic, S. et al (2004) Adv Cancer Res 95:1-30). Eighty percent of synovial sarcomas express NY-ESO-1 (Jungbluth, A. et al (2001) In J. Cancer 94:252-256).

[0004] Spontaneous CD8 and CD4 T-cell responses to NY-ESO-1 in cancer patients have been identified and studied (Jager, E. et al (1998) J Exp Med 187:265-270; Jager, E. et al (2000) J Exp Med 191:628-630). NY-ESO-1 peptides 157-165, 157-167 and 155-163 are restricted by HLA-A2 in tumor reactive T cell CD8 lines, and peptide 56-62 is also recognized in HLA-A31 CD8 T cells (Jager et al (1998) J. Exp Med 187:265-270; Wang, R.F. et al (1998) J. Immunol 161:3598-3606). Several epitopes restricted by HLA-DR4 in CD4 T cell responses have been demonstrated, and responses against peptide 157-170 were restricted by HLA-DP4, an allele found in the majority of Caucasians (Jager et al (2000) J Exp Med 191:625-630; Zang et al (2001) Proc Natl Acad Sci 98:3964-3969).

[0005] The NY-ESO-1 antigen has been evaluated in immunotherapy as a cancer vaccine candidate, including based on full length protein, various peptide fragments, DNA, and recombinant virus-based immunogens. Analysis of CD8⁺ T cells generated in vaccine trials using NY-ESO-1 derived peptides (157-165 and 157-167) showed that the dominant immune response was directed against a cryptic epitope (159-167), which diverted the immune response from tumor recognition. CTLs reactive to the NY-ESO-1₁₅₇₋₁₆₅ peptide were capable of lysing NY-ESO-1/HLA-A0201-expressing tumor cells. Therefore, a ligand binding specifically to NY-ESO-1₁₅₇₋₁₆₅/HLA complex has been recognized as a potentially useful cancer agent.

[0006] Phage display technology was utilized to isolate Fab antibodies with T-cell receptor-like specificity, and Fab Abs recognizing the NY-ESO-1₁₅₇₋₁₆₅ peptide in the HLA-A0201 context were isolated. Nine different antibodies were identified with TCR-like specificity and all bound specifically to NY-ESO-1₁₅₇₋₁₆₅/HLA-2 complexed by ELISA or FACS analysis on the surface of peptide-pulsed T2 cells. Representative clones included 3M4E5, 2M4B2, 3M4H7, and 2M4D10 (Held G. et al (2004) Eur J Immunol 34:2919-2929). Clone 3M4E5 was initially chosen for further analysis because it displayed a substantial shift in FACS analysis. Fab-3M4E5 binds to the NY-ESO-1₁₅₇₋₁₆₅/HLA-2 complex displayed on mammalian cells after endogenous processing and blocks T-cell reactivity to the epitope NY-ESO-1₁₅₇₋₁₆₅ displayed by target cells in the HLA-A2 context. Melanoma cell line SK-MEL-37, which expresses

both HLA-A2 and NY-ESO-1, is stained by Fab-3M4E5 only weakly (Held, G. et al (2004) Eur J Immunol 34:2919-2929).

[0007] Thus, while the extant evidence of activity of NY-ESO-1 antibodies is encouraging, the observed limitations on affinity remain. Accordingly, it would be desirable to develop antibodies, particularly including T cell receptor-like antibodies or fragments and like agents that demonstrate enhanced affinity and increased efficacy and applicability in cancer diagnosis and therapy, and it is toward the achievement of that objective that the present invention is directed.

[0008] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0009] The invention provides affinity matured high affinity antibodies mimicking a TCR that are useful in targeting specific pMHC complexes for diagnostic and therapeutic purposes. In particular, high affinity antibodies targeting NY-ESO-1 peptide MHC complexes are provided, wherein said antibodies have a peptideMHC affinity which is greater than that of corresponding TCRs. Fab antibodies and single chain antibodies are particularly provided herein. The antibodies of the present invention have diagnostic and therapeutic use and application in cancers, particularly in cancers wherein NY-ESO-1 is involved or otherwise presented during the cancer or metastatic process or is a part of the humoral and/or cellular response to cancer, oncogenesis, or other disease pathogenesis. In a particular aspect the antibodies of the invention are applicable in cancers, including melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma.

[00010] In a general aspect, the present invention provides T cell receptor-like antibodies directed against NY-ESO-1 peptide antigen(s) in complex with MHC molecule(s) and having affinity for peptideMHC complexes which is greater than the relative affinity of T cell receptor(s). In a broad aspect, the present invention provides an isolated specific binding member, particularly an antibody or fragment thereof, including an Fab fragment and a single chain or domain antibody, which recognizes an epitope on NY-ESO-1 antigen in combination with MHC peptide. In a further aspect, the present invention provides an antibody or fragment thereof, which recognizes NY-ESO-1 peptide 157-165/Class I MHC epitope which is found in tumorigenic, hyperproliferative or abnormal cells and is not detectable in normal somatic cells. The NY-ESO-1₁₅₇₋₁₆₅ immunodominant peptide corresponds in sequence to SLLMWITQV.

[00011] In a particular aspect, the antibody or fragment of the invention is specific for NY-ESO-1 peptide MHC molecules and is a T cell receptor like antibody, recognizing peptideMHC molecules with affinity that is greater than that of the T-cell receptor (TCR). In one aspect, the antibody of the invention has a binding affinity for NY-ESO-1 peptide MHC molecules, particularly for NY-ESO-1₁₅₇₋₁₆₅ /HLA complex, of less than 10 nm. In a particular such aspect, the affinity of antibodies of the present invention for NY-ESO-1₁₅₇₋₁₆₅ /HLA-A*0201 target is on the order of 2-4nm. In one such aspect, the affinity of antibodies of the present invention for NY-ESO-1₁₅₇₋₁₆₅ /HLA-A*0201 target exceeds the affinity of TCR by about 1000 fold.

[00012] The present inventors have discovered novel high affinity T cell receptor-like antibodies, exemplified herein by the antibodies designated T1, T2 and T3, which specifically and with high affinity recognize NY-ESO-1 peptide MHC complexes, particularly NY-ESO-1 peptide 157-165/MHC molecules, particularly NY-ESO-1₁₅₇₋₁₆₅ /HLA-A*0201. The antibodies exemplified herein include Fab antibodies and single chain antibodies based thereon. The antibodies have the amino acid sequences as set out herein and in Figures 10, 12 and 13.

[00013] The antibodies of the present invention can specifically categorize the nature of cancer and tumor cells, by binding, staining or otherwise recognizing those cells wherein NY-ESO-1 peptide MHC complexes, particularly NY-ESO-1 peptide 157-165/MHC molecules, is present or presented for immune system recognition and modulation. Further, the antibodies of the present invention, as exemplified by antibodies T1, T2 and T3, when converted into scFv fragments and cloned into vectors to generate recombinant single chain TCRs, demonstrate specific recognition and killing of T cells presenting the NY-ESO-1₁₅₇₋₁₆₅ peptide and not others.

[00014] The unique specificity and high affinity of the antibodies and fragments of the invention provides diagnostic and therapeutic uses to identify, characterize and target a number of tumor types, for example, melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma, without the problems associated with normal tissue uptake.

[00015] In a preferred aspect, the antibody is one which has the characteristics of the antibody which the inventors have identified and characterized, in particular recognizing NY-ESO-1 peptide MHC complexes, particularly NY-ESO-1 peptide 157-165/MHC molecules. In a particularly preferred aspect

the antibody is T1, T2 or T3, or active fragments thereof. In a further preferred aspect the antibody of the present invention comprises the VH and VL amino acid sequences depicted in Figures 10, 12 and 13. In a particular aspect, the antibody of the invention comprises the CDR sequences depicted in Figure 10, Figure 12 or Figure 13. In a particular aspect of the invention the antibody is T1 and comprises the variable region sequences set out in Figure 10. In a particular aspect of the invention the antibody is T1 and comprises the CDR region sequences set out in Figure 10.

[00016] The binding of an antibody to its target antigen is mediated through the complementarity-determining regions (CDRs) of its heavy and light chains. Accordingly, specific binding members based on the CDR regions of the heavy or light chain, and preferably both, of the antibodies of the invention, particularly of T1, T2, and/or T3, will be useful specific binding members for therapy and/or diagnostics. The CDRs of the antibodies are depicted in Figures 10, 12 and 13. Antibody T1 comprises heavy chain CDR sequences GFTFSTY, IVSSGGST and AGELLPYYGMDV and light chain CDR sequences ERDVGGNY, DVI and WSFAGGYV, as set out in Figure 10. Antibody T2 comprises heavy chain CDR sequences GFTFSTY, ILSSGGET and AGMLLPYYGMDV and light chain CDR sequences ERDVGGNY, DVI and WSFAGGYV, as set out in Figure 12. Antibody T3 comprises heavy chain CDR sequences GFTFSTY, IASSGGET and AGSLLPYYGMDV and light chain CDR sequences ERDVGGNY, DVI and WSFAGGYV, as set out in Figure 13.

[00017] Accordingly, specific binding proteins such as antibodies which are based on the CDRs of the antibody(ies) identified herein will be useful for targeting NY-ESO-1 cancers and/or NY-ESO-1/MHC complexes, and/or NY-ESO-1 peptides presented by antigen presenting cells. As the antibodies of the invention do not bind significantly to normal somatic cells or to cells presenting alternative or distinct antigens, and have peptide MHC affinities greater than TCRs, it is anticipated that there will not be significant uptake in normal tissue and there will be suitable affinity for the complexes, a limitation of other antibodies.

[00018] In a further aspect, the present invention provides an isolated antibody or fragment thereof capable of binding an antigen, wherein said antibody or fragment thereof comprises a polypeptide binding domain comprising an amino acid sequence substantially as set out in Figures 10, 12 and 13.

[00019] In further aspects, the invention provides an isolated nucleic acid which comprises a sequence encoding a specific binding member as defined above, and methods of preparing specific binding

members of the invention which comprise expressing said nucleic acids under conditions to bring about expression of said binding member, and recovering the binding member. In one such aspect, a nucleic acid encoding antibody variable region sequence having the amino acid sequences as set out in Figures 10, 12 or 13 is provided or an antibody having CDR domain sequences as set out in Figures 10, 12 or 13 is provided. In one aspect, a nucleic acid of Figure 11 is provided. The present invention also relates to a recombinant DNA molecule or cloned gene, or a degenerate variant thereof, which encodes an antibody of the present invention; preferably a nucleic acid molecule, in particular a recombinant DNA molecule or cloned gene, encoding the antibody VH and VL, particularly the CDR region sequences, which has a sequence or is capable of encoding a sequence shown in FIGURE 10, 12 or 13.

[00020] The antibodies, fragments thereof and recombinant single chain TCRs according to the invention may be used in a method of treatment or diagnosis of the human or animal body, such as a method of treatment of a tumor in a human patient which comprises administering to said patient an effective amount of the antibodies, fragments thereof and recombinant single chain TCRs of the invention.

[00021] The present invention also includes polypeptides or antibodies having the activities noted herein, and that display the amino acid sequences set forth and described above and in Figures 10, 12 or 13 hereof, or are antibodies having a heavy chain and a light chain wherein the complementarity determining regions (CDRs) of the heavy and light chain comprise the amino acid sequences depicted in each or any of Figures 10, 12 and 13.

[00022] The diagnostic utility of the present invention extends to the use of the antibodies of the present invention in assays to characterize tumors or cellular samples or to screen for tumors or cancer, including *in vitro* and *in vivo* diagnostic assays. In an immunoassay, a control quantity of the antibodies, or the like may be prepared and labeled with an enzyme, a specific binding partner and/or a radioactive element, and may then be introduced into a cellular sample. After the labeled material or its binding partner(s) has had an opportunity to react with sites within the sample, the resulting mass may be examined by known techniques, which may vary with the nature of the label attached.

[00023] Specific binding members of the invention may carry a detectable or functional label. The specific binding members may carry a radioactive label, such as the isotopes ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{121}I , ^{124}I , ^{125}I , ^{131}I , ^{111}In , ^{117}Lu , ^{211}At , ^{198}Au , ^{67}Cu , ^{225}Ac , ^{213}Bi , ^{99}Tc and ^{186}Re . When radioactive labels are used, known currently available counting procedures may be utilized to identify and quantitate the specific binding members. In the instance where the label is an enzyme, detection may be

accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

[00024] The radiolabelled specific binding members, particularly antibodies and fragments thereof, are useful in *in vitro* diagnostics techniques and in *in vivo* radioimaging techniques. In a further aspect of the invention, radiolabelled specific binding members, particularly antibodies and fragments thereof, particularly radioimmunoconjugates, are useful in radioimmunotherapy, particularly as radiolabelled antibodies for cancer therapy. In a still further aspect, the radiolabelled specific binding members, particularly antibodies and fragments thereof, are useful in radioimmuno-guided surgery techniques, wherein they can identify and indicate the presence and/or location of cancer cells, precancerous cells, tumor cells, and hyperproliferative cells, prior to, during or following surgery to remove such cells.

[00025] Immunoconjugates or antibody fusion proteins of the present invention, wherein the specific binding members, particularly antibodies and fragments thereof, of the present invention are conjugated or attached to other molecules or agents further include, but are not limited to binding members conjugated to a chemical ablation agent, toxin, immunomodulator, cytokine, cytotoxic agent, chemotherapeutic agent or drug.

[00026] The present invention includes an assay system which may be prepared in the form of a test kit for the quantitative analysis of the extent of the presence of, for instance, NY-ESO-1 peptide/MHC complexes. The system or test kit may comprise a labeled component prepared by one of the radioactive and/or enzymatic techniques discussed herein, coupling a label to the antibody, and one or more additional immunochemical reagents, at least one of which is a free or immobilized components to be determined or their binding partner(s).

[00027] In a further embodiment, the present invention relates to certain therapeutic methods which would be based upon the activity of the binding member, antibody, or active fragments thereof, or upon agents or other drugs determined to possess the same activity. A first therapeutic method is associated with the prevention or treatment of cancer, including but not limited to melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma.

[00028] The binding members and antibodies of the present invention, and in a particular embodiment the antibody whose sequences are presented in Figure 10, 12 and 13 herein, or active fragments thereof, and single chain, recombinant or synthetic antibodies derived therefrom, particularly

comprising the CDR region sequences depicted in Figures 10, 12 and 13, can be prepared in pharmaceutical compositions, including a suitable vehicle, carrier or diluent, for administration in instances wherein therapy is appropriate, such as to treat cancer. Such pharmaceutical compositions may also include methods of modulating the half-life of the binding members, antibodies or fragments by methods known in the art such as pegylation. Such pharmaceutical compositions may further comprise additional antibodies or therapeutic agents.

[00029] A composition of the present invention may be administered alone or in combination with other treatments, therapeutics or agents, either simultaneously or sequentially dependent upon the condition to be treated. In addition, the present invention contemplates and includes compositions comprising the binding member, particularly antibody or fragment thereof, herein described and other agents or therapeutics such as anti-cancer agents or therapeutics, anti-mitotic agents, apoptotic agents or antibodies, or immune modulators. More generally these anti-cancer agents may be tyrosine kinase inhibitors or phosphorylation cascade inhibitors, post-translational modulators, cell growth or division inhibitors (*e.g.* anti-mitotics), inhibitors or signal transduction inhibitors. Other treatments or therapeutics may include the administration of suitable doses of pain relief drugs such as non-steroidal anti-inflammatory drugs (*e.g.* aspirin, paracetamol, ibuprofen or ketoprofen) or opiates such as morphine, or anti-emetics. In addition, the composition may be administered with immune modulators, such as interleukins, tumor necrosis factor (TNF) or other growth factors, colony stimulating factors, cytokines or hormones such as dexamethasone which stimulate the immune response and reduction or elimination of cancer cells or tumors. The composition may also be administered with, or may include combinations along with other anti-NY-ESO-1 antibodies or other anti-tumor antigen antibodies.

[00030] The present invention also includes antibodies and fragments thereof, which are covalently attached to or otherwise associated with other molecules or agents. These other molecules or agents include, but are not limited to, molecules (including antibodies or antibody fragments) with distinct recognition characteristics, toxins, ligands, and chemotherapeutic agents.

[00031] Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing detailed description, which proceeds with reference to the following illustrative drawings, and the attendant claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00032] FIGURE 1A-1F. Structural comparison of TCR and Fab. **(A)** Comparison of positions of 1G4 TCR (red) and 3M4E5 Fab in complex with HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₇. **(B)** Positions of the CDR3 loops (blue and magenta) bound to the peptide (cyan) and MHC (grey), and the two positions of the CDR2 VH loop from the 3M4F4 complex in the up (red) and down conformations. **(C)** Schematic of CDR loops of 1G4 TCR in complex with HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₇ with loops coloured as follows: V α CDR1, green; V α CDR2, red; V α CDR3, blue; V β CDR1, magenta; V β CDR2, orange; V β CDR3, cyan. Water molecules within 4Å of both TCR and peptide are indicated as red spheres. **(D)** Diagram of CDR loops of 3M4E5 Fab in complex with HLA- A*0201/NYESO-1₁₅₇₋₁₆₇ with loops coloured as follows: VH CDR1, green; VH CDR2, red; VH CDR3, blue; VL CDR1, magenta; VL CDR2, orange; VL CDR3, cyan. Water molecules within 4Å of both TCR and peptide are indicated as red spheres. **(E)** Structure of the 1G4 TCR residues forming the 'roof' residues in the cavity that binds the peptide (cyan) MW side chains. **(F)** The analogous 3M4E5 Fab side chains that form the peptide MW binding cavity. Hydrogen bonds are shown in black dashed lines.

[00033] FIGURE 2. Simulated annealing omit electron density map showing the peptide MW residues bound by the 3M4F5 Fab in yellow chicken wire. Fab residues are coloured according to the CDR colour as in Fig. 1D. Hydrogen bonds between the Fab and the MW residues are shown in black. The map is contoured at 3 σ .

[00034] FIGURE 3A-3C. **(A)** Superposition, based on the MHC α 1/ α 2 helices, of the two 3M4E5 and four 3M4F4 Fab complexes found in the crystallographic asymmetric unit, illustrating the variable positioning of the Fabs in the different crystal contexts. Colours are for 3M4E5 chains H/L, green; K/M, red; and for 3M4F4 H/L, blue; G/I, magenta; N/O, cyan and S/T coral (chain labels correspond to those for the deposited PDB files). MHC is coloured grey and the NYESO-1₁₅₇₋₁₆₅ peptide in yellow. **(B)** Superposition, based on the VH domains, of all 6 bound Fab structures (3M4E5 chains H and K, 3M4F4 chains H, I, O, T), illustrating the 'down' conformation of the CDR2 VH loop found in chains H and I of the 3M4F4 Fab, where in all the other Fab structures, the CDR2 adopts the 'up' conformation. **(C)** Superposition of the VH domains from representative 3M4F4 structures for the two CDR2 VH conformations, chains H (blue) and T (coral), illustrating the 7Å main chain change and side chain reorientations.

[00035] FIGURE 4A-4F. Conformational changes on binding for **(A)** the 1G4 TCR (alpha chain is green and beta chain is red, unliganded structures in light colours) and **(B)** the 3M4F5 Fab (heavy chain is green and light chain is red, unliganded structures in light colours) where minor conformational

adjustments are apparent for the 1G4 TCR compared to almost no significant main chain conformational adjustment of the 3M4E5 Fab CDR loops. The peptide MW residues are illustrated in yellow. Molecular surfaces (coloured as above) for the liganded (**C**) and unliganded (**E**) 1G4 TCR, and the liganded (**D**) and unliganded (**F**) 3M4E5. For both, 1G4 and 3M4E5, a cavity is preformed in the unliganded structures that can accommodate the peptide MW side chains (**E** and **F**).

[00036] FIGURE 5A-5D. Structural basis for a second generation library. (**A**) Side view of 3M4E5 Fab in complex with HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₇, with residues randomised in the second-generation phage display library highlighted as spheres. Colours are as in figure 1d. (**B-D**) Illustrations of 3M4E5 Fab CDR residues that were randomized (red) in the vicinity of the peptide which displayed non-optimal contacts with the peptide. Residues making key contacts with the peptide MW side chains and lining the cavity in the CDR loops that interacts with the MW peg are coloured in blue. The NYESO-1₁₅₇₋₁₆₇ peptide is coloured cyan, and hydrogen bonds represented as dashed black lines.

[00037] FIGURE 6. Classification of 3M4E5 mutants by phylogenetic tree analysis. Amino acid sequences of mutated regions were subjected to cluster analysis using CLUSTAL W software for 123 mutant clones. The following conditions were used: matrix, BLOSUM; gap opening penalty, 10.0; gap extension penalty, 0.05. Results are displayed as a phylogenetic tree, which indicates the genetic distance to the parental 3M4E5 sequence. The three types of mutants that were repeatedly identified are indicated in red.

[00038] FIGURE 7A-7D. Binding characteristics of 3M4E5-evolved Fab antibodies T1-3. (**A**) Comparison of binding characteristics as detected by ELISA on HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complexes. Fab Type1 T1(●), Type2 T2(◆), Type3 T3(▲), control Fab(△) with weak binding characteristics and Fab wt 3M4E5 (□) were titrated over the indicated concentration range. (**B**) Confirmation of Fab binding specificity was achieved by incubation with minigene transfected T2 cells. Reactivity of 3M4E5 and Fabs T1-T3 molecules with target HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complex (white column) and control complexes (black HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₇ and grey HLA-A*0201/NY-ESO-1₁₅₅₋₁₆₃) is depicted. HLA-A*0201 expression was confirmed for all cell lines by BB7.2 and w6/32 antibody staining, respectively. (**C**) Inhibition of 3M4E5-b binding by competition FACS analysis. Co-incubation of PE-labeled biotinylated 3M4E5-b-tetramers and different concentrations (50, 5, 0.5, 0.05, 0.005 0.0005, 0µg/ml) of non-labeled 3M4E5 (□), T1 (●), T2 (◆), T3 (▲) or control Fab (△) with peptide pulsed T2 cells. (**D**) Inhibition of NY-ESO-1₁₅₇₋₁₆₅-specific CTL responses by indicated Fab concentrations of T1 (●), Fab 3M4E5 (□) or irrelevant Fab (△). IFN-γ response of NY-ESO-1₁₅₇₋₁₆₅-specific CTL to T2

cells pulsed with SLLMWITQC peptide (10^{-6} M) was detected by ELISPOT. All assays were done in triplicate.

[00039] **FIGURE 8.** Tetramer staining of transduced T-cells. Binding specificity of T1(●), wt (■) and control (▲) sc-TCRs on transduced CD3⁺ T-cells were analysed by incubation with indicated amounts of PE conjugated HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ tetramers using flow cytometry. Assay was done in triplicates and standard deviation is depicted.

[00040] **FIGURE 9.** Fab activity as recombinant scTCRs. Specific lysis of HLA-A*0201 positive T2 cells expressing either the NY-ESO-1₁₅₇₋₁₆₅ (A) or IMP₅₈₋₆₆ Flu peptide (B) by indicated amounts of receptor-grafted T cells [T1 (●), 3M4E5 (□), anti-CEA scTCR (△)] was determined colorimetrically as described. All assays were performed at least in triplicate with T1 always achieving a significantly ($p < 0.05$) higher cytotoxic activity.

[00041] **FIGURE 10** depicts the amino acid sequence of the heavy and light chain variable region of antibody T1 compared with antibody 3M4E5. The CDR regions are depicted in blue. Amino acid differences are shown in red.

[00042] **FIGURE 11** depicts the nucleic acid sequence encoding the heavy and light chain variable region of antibody T1 compared with antibody 3M4E5. The CDR regions are depicted in blue. Nucleotide differences are shown in red.

[00043] **FIGURE 12** depicts the amino acid sequence of the heavy and light chain variable region of antibody T2 compared with antibody 3M4E5. The CDR regions are depicted in blue. Amino acid differences are shown in red.

[00044] **FIGURE 13** depicts the amino acid sequence of the heavy and light chain variable region of antibody T3 compared with antibody 3M4E5. The CDR regions are depicted in blue. Amino acid differences are shown in red.

DETAILED DESCRIPTION

[00045] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual"

(1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

[00046] Therefore, if appearing herein, the following terms shall have the definitions set out below.

A. TERMINOLOGY

[00047] The term "specific binding member" describes a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its surface, or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate. This application is concerned with antigen-antibody type reactions.

[0001] The term "antibody" describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. CDR grafted antibodies are also contemplated by this term. An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. The term "antibody(ies)" includes a wild type immunoglobulin (Ig) molecule, generally comprising four full length polypeptide chains, two heavy (H) chains and two light (L) chains, or an equivalent Ig homologue thereof (e.g., a camelid nanobody, which comprises only a heavy chain); including full length functional mutants, variants, or derivatives thereof, which retain the essential epitope binding features of an Ig molecule, and including dual specific, bispecific, multispecific, and dual variable domain antibodies; Immunoglobulin molecules can be of any class (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), or subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2). Also included within the meaning of the term "antibody" are any "antibody fragment".

[0002] An "antibody fragment" means a molecule comprising at least one polypeptide chain that is not full length, including (i) a Fab fragment, which is a monovalent fragment consisting of the variable light (VL), variable heavy (VH), constant light (CL) and constant heavy 1 (CH1) domains; (ii) a F(ab')₂ fragment, which is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a heavy chain portion of an Fab (Fd) fragment, which consists of the VH and CH1 domains; (iv) a variable fragment (Fv) fragment, which consists of the VL and VH domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment, which comprises a single variable domain (Ward, E.S. et al., *Nature* 341, 544-546 (1989)); (vi) a camelid antibody; (vii) an isolated complementarity determining region (CDR); (viii) a Single Chain Fv Fragment wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, *Science*, 242, 423-426, 1988; Huston et al, *PNAS USA*, 85, 5879-5883, 1988); (ix) a diabody, which is a bivalent, bispecific antibody in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with the complementarity domains of another chain and creating two antigen binding sites (WO94/13804; P. Holliger et al *Proc. Natl. Acad. Sci. USA* 90 6444-6448, (1993)); and (x) a linear antibody, which comprises a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementarity light chain polypeptides, form a pair of antigen binding regions; (xi) multivalent antibody fragments (scFv dimers, trimers and/or tetramers (Power and Hudson, *J Immunol. Methods* 242: 193-204 9 (2000)); and (xii) other non-full length portions of heavy and/or light chains, or mutants, variants, or derivatives thereof, alone or in any combination.

[00048] As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023 and U.S. Patent Nos. 4,816,397 and 4,816,567.

[00049] An "antibody combining site" is that structural portion of an antibody molecule comprised of light chain or heavy and light chain variable and hypervariable regions that specifically binds antigen.

[00050] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[00051] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v), which portions are preferred for use in the therapeutic methods described herein.

[00052] Antibodies may also be bispecific, wherein one binding domain of the antibody is a specific binding member of the invention, and the other binding domain has a different specificity, *e.g.* to recruit an effector function or the like. Bispecific antibodies of the present invention include wherein one binding domain of the antibody is a specific binding member of the present invention, including a fragment thereof, and the other binding domain is a distinct antibody or fragment thereof, including that of a distinct anti-cancer or anti-tumor specific antibody. The other binding domain may be an antibody that recognizes or targets a particular cell type, as in a neural or glial cell-specific antibody. In the bispecific antibodies of the present invention the one binding domain of the antibody of the invention may be combined with other binding domains or molecules which recognize particular cell receptors and/or modulate cells in a particular fashion, as for instance an immune modulator (*e.g.*, interleukin(s)), a growth modulator or cytokine (*e.g.* tumor necrosis factor (TNF), and particularly, the TNF bispecific modality demonstrated in U.S.S.N. 60/355,838 filed February 13, 2002 incorporated herein in its entirety) or a toxin (*e.g.*, ricin) or anti-mitotic or apoptotic agent or factor.

[00053] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may also contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; *e.g.*, a bispecific (chimeric) monoclonal antibody.

[00054] The term "antigen binding domain" describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may bind to a particular part of the antigen only, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an

antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

[00055] Immunoconjugates or antibody fusion proteins of the present invention, wherein the antibodies, antibody molecules, or fragments thereof, of use in the present invention are conjugated or attached to other molecules or agents further include, but are not limited to such antibodies, molecules, or fragments conjugated to a chemical ablation agent, toxin, immunomodulator, cytokine, cytotoxic agent, chemotherapeutic agent, antimicrobial agent or peptide, cell wall and/or cell membrane disrupter, or drug.

[00056] The term “specific” may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner(s). The term is also applicable where *e.g.* an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific binding member carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

[00057] The term “comprise” generally used in the sense of include, that is to say permitting the presence of one or more features or components.

[00058] The term “consisting essentially of” refers to a product, particularly a peptide sequence, of a defined number of residues which is not covalently attached to a larger product. In the case of the peptide of the invention referred to above, those of skill in the art will appreciate that minor modifications to the N- or C- terminal of the peptide may however be contemplated, such as the chemical modification of the terminal to add a protecting group or the like, *e.g.* the amidation of the C-terminus.

[00059] The term “isolated” refers to the state in which specific binding members of the invention, or nucleic acid encoding such binding members will be, in accordance with the present invention. Members and nucleic acid will be free or substantially free of material with which they are naturally associated such as other polypeptides or nucleic acids with which they are found in their natural environment, or the environment in which they are prepared (*e.g.* cell culture) when such preparation is by recombinant DNA technology practised *in vitro* or *in vivo*. Members and nucleic acid may be formulated with diluents or adjuvants and still for practical purposes be isolated - for example the members will normally be mixed with gelatin or other carriers if used to coat microtitre plates for use in immunoassays, or will be mixed with pharmaceutically acceptable carriers or diluents when used in diagnosis or therapy.

[00060] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

[00061] The terms "antibody", "T cell receptor-like antibody", "NY-ESO-1 peptide/MHC antibody", "antibody T1", "antibody T2", "antibody T3" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in Figures 10, 12 and 13 and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "antibody", "T cell receptor-like antibody", "NY-ESO-1 peptide/MHC antibody", "antibody T1", "antibody T2", "antibody T3" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

[00062] The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, **243**:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

<u>SYMBOL</u>		<u>AMINO ACID</u>
<u>1-Letter</u>	<u>3-Letter</u>	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine

L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
C	Cys	cysteine

[00063] It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

[00064] A "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

[00065] A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

[00066] A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (*e.g.*, restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

[00067] An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

[00068] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[00069] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[00070] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

[00071] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[00072] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the

host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[00073] The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

[00074] The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[00075] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[00076] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

[00077] A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for

example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

[00078] Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, *e.g.*, Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

[00079] It should be appreciated that also within the scope of the present invention are DNA sequences encoding specific binding members (antibodies) of the invention which code for *e.g.* an antibody having the same amino acid sequence as provided in Figure 10, 12 or 13, or comprising the CDR domain region sequences set out in Figures 10, 12 or 13 but which are degenerate thereto. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

Phenylalanine (Phe or F)	UUU or UUC
Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
Isoleucine (Ile or I)	AUU or AUC or AUA
Methionine (Met or M)	AUG
Valine (Val or V)	GUU or GUC or GUA or GUG
Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
Proline (Pro or P)	CCU or CCC or CCA or CCG
Threonine (Thr or T)	ACU or ACC or ACA or ACG
Alanine (Ala or A)	GCU or GCG or GCA or GCG
Tyrosine (Tyr or Y)	UAU or UAC
Histidine (His or H)	CAU or CAC

Glutamine (Gln or Q)	CAA or CAG
Asparagine (Asn or N)	AAU or AAC
Lysine (Lys or K)	AAA or AAG
Aspartic Acid (Asp or D)	GAU or GAC
Glutamic Acid (Glu or E)	GAA or GAG
Cysteine (Cys or C)	UGU or UGC
Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
Glycine (Gly or G)	GGU or GGC or GGA or GGG
Tryptophan (Trp or W)	UGG
Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)

[00080] It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

[00081] Mutations can be made in the sequences encoding the amino acids set out in Figures 10, 12 or 13, or in the sequence of Figure 11, such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (*i.e.*, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (*i.e.*, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein.

[00082] The following is one example of various groupings of amino acids:

Amino acids with nonpolar R groups

Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine

Amino acids with uncharged polar R groups

Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine

Amino acids with charged polar R groups (negatively charged at Ph 6.0)

Aspartic acid, Glutamic acid

Basic amino acids (positively charged at pH 6.0)

Lysine, Arginine, Histidine (at pH 6.0)

[00083] Another grouping may be those amino acids with phenyl groups:

Phenylalanine, Tryptophan, Tyrosine

[00084] Another grouping may be according to molecular weight (*i.e.*, size of R groups):

Glycine	75	Alanine	89
Serine	105	Proline	115
Valine	117	Threonine	119
Cysteine	121	Leucine	131
Isoleucine	131	Asparagine	132
Aspartic acid	133	Glutamine	146
Lysine	146	Glutamic acid	147
Methionine	149	Histidine (at pH 6.0)	155
Phenylalanine	165	Arginine	174
Tyrosine	181	Tryptophan	204

[00085] Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH₂ can be maintained.

[00086] Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β -turns in the protein's structure.

[00087] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[00088] A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (*e.g.*, a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[00089] A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (*e.g.*, ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[00090] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

[00091] The term 'agent' means any molecule, including polypeptides, antibodies, polynucleotides, chemical compounds and small molecules. In particular the term agent includes compounds such as test compounds or drug candidate compounds.

[00092] The term 'agonist' refers to a ligand that stimulates the receptor the ligand binds to in the broadest sense.

[00093] The term ‘assay’ means any process used to measure a specific property of a compound. A ‘screening assay’ means a process used to characterize or select compounds based upon their activity from a collection of compounds.

[00094] The term ‘preventing’ or ‘prevention’ refers to a reduction in risk of acquiring or developing a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop) in a subject that may be exposed to a disease-causing agent, or predisposed to the disease in advance of disease onset.

[00095] The term ‘prophylaxis’ is related to and encompassed in the term ‘prevention’, and refers to a measure or procedure the purpose of which is to prevent, rather than to treat or cure a disease. Non-limiting examples of prophylactic measures may include the administration of vaccines; the administration of low molecular weight heparin to hospital patients at risk for thrombosis due, for example, to immobilization; and the administration of an anti-malarial agent such as chloroquine, in advance of a visit to a geographical region where malaria is endemic or the risk of contracting malaria is high.

[00096] ‘Therapeutically effective amount’ means that amount of a drug, compound, antimicrobial, antibody, or pharmaceutical agent that will elicit the biological or medical response of a subject that is being sought by a medical doctor or other clinician. In particular, with regard to gram-positive bacterial infections and growth of gram-positive bacteria, the term “effective amount” is intended to include an effective amount of a compound or agent that will bring about a biologically meaningful decrease in the amount of or extent of infection of gram-positive bacteria, including having a bacteriocidal and/or bacteriostatic effect. The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in the growth or amount of infectious bacteria, or other feature of pathology such as for example, elevated fever or white cell count as may attend its presence and activity.

[00097] The term ‘treating’ or ‘treatment’ of any disease or infection refers, in one embodiment, to ameliorating the disease or infection (i.e., arresting the disease or growth of the infectious agent or bacteria or reducing the manifestation, extent or severity of at least one of the clinical symptoms thereof). In another embodiment ‘treating’ or ‘treatment’ refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, ‘treating’ or ‘treatment’ refers to modulating the disease or infection, either physically, (e.g., stabilization of a discernible symptom),

physiologically, (e.g., stabilization of a physical parameter), or both. In a further embodiment, 'treating' or 'treatment' relates to slowing the progression of a disease or reducing an infection.

[00098] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[00099] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

B. DETAILED DISCLOSURE.

[000100] The present invention provides novel high affinity antibodies mimicking a TCR that are useful in targeting specific pMHC complexes for diagnostic and therapeutic purposes. In particular, high affinity antibodies targeting NY-ESO-1 peptide MHC complexes are provided, wherein said antibodies have a peptideMHC affinity which is greater than that of corresponding TCRs. The invention provides an antibody or fragment thereof, which recognizes NY-ESO-1 peptide 157-165/MHC complexes with high and specific affinity. The antibodies of the present invention have diagnostic and therapeutic use and application in cancers, particularly in cancers wherein NY-ESO-1 is involved or otherwise presented during the cancer or metastatic process or is a part of the humoral and/or cellular response to cancer, oncogenesis, or other disease pathogenesis. In a particular aspect the antibodies of the invention are applicable in cancers, including melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma.

[000101] The unique specificity of the antibodies or fragments thereof of the present invention, recognizing tumor antigen peptide/MHC complexes, particularly NY-ESO-1/MHC complexes, with specificity and with greater affinity than TCRs, provides diagnostic and therapeutic in cancer and immune-mediated cancer therapy. The antibodies of the present invention can thus specifically categorize the nature of tumors or tumorigenic cells, by staining or otherwise recognizing those tumors or cells wherein NY-ESO-1 or NY-ESO-1/MHC complexes are present or presented in or on cells.

[000102] Panels of monoclonal antibodies recognizing NY-ESO-1 or NY-ESO-1/MHC complexes can be screened for various properties; *i.e.*, isotype, epitope, affinity, etc. Of particular interest are

antibodies that mimic the activity of exemplary antibodies T1, T2 and T3, and have affinity for NY-ESO-1/MHC complexes which is greater than that of TCRs. Such antibodies can be readily identified and/or screened in specific binding member activity assays.

[000103] In general, the CDR regions, comprising amino acid sequences substantially as set out as the CDR regions of Figures 10, 12 and 13 will be carried in a structure which allows for binding of the CDR regions to an tumor antigen, particularly to a tumor antigen peptide/MHC complex in a T cell receptor-like fashion.

[000104] By "substantially as set out" it is meant that that variable region sequences, and/or particularly the CDR sequences, of the invention will be either identical or highly homologous to the specified regions of Figure 10, 12 or 13. By "highly homologous" it is contemplated that only a few substitutions, preferably from 1 to 8, preferably from 1 to 5, preferably from 1 to 4, or from 1 to 3, or 1 or 2 substitutions may be made in the variable region sequence and/or in the CDR sequences.

[000105] Substitutions may be made in the variable region sequence outside of the CDRs so as to retain the CDR sequences. Thus, changes in the variable region sequence or alternative non-homologous or veneered variable region sequences may be introduced or utilized, such that the CDR sequences are maintained and the remainder of the variable region sesuence may be substituted. Alternatively, substitutions may be made particularly in the CDRs. CDR sequences for the antibodies of the present invention are set out herein including in Figures 10, 12 and 13. Particularly preferred amino acids for substitution are VL residues 26S, 27R, 32Y, 95G, 96S, 97Y and VH Q33, S35, G50, V52, S57, A59, E99, as set out herein and in the figures.

[000106] Antibodies of the invention having substitutions as above described and contemplated are selected to maintain the activities, specificity, and affinity commensurate with or greater than the exemplary antibodies, including T1, T2 and T3 and having the characteristics as set out herein and in the claims.

[000107] The structure for carrying the CDRs of the invention will generally be of an antibody heavy or light chain sequence or substantial portion thereof in which the CDR regions are located at locations corresponding to the CDR region of naturally occurring VH and VL antibody variable domains encoded by rearranged immunoglobulin genes. The structures and locations of immunoglobulin variable domains may be determined by reference to Kabat, E.A. *et al*, Sequences of Proteins of Immunological Interest. 4th

Edition. US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (<http://immuno.bme.nwu.edu>)).

[000108] The variable domains may be derived from any germline or rearranged human variable domain, or may be a synthetic variable domain based on consensus sequences of known human variable domains. The CDR-derived sequences of the invention, as defined in the preceding paragraph, may be introduced into a repertoire of variable domains lacking CDR regions, using recombinant DNA technology.

[000109] For example, Marks *et al* (Bio/Technology, 1992, 10:779-783) describe methods of producing repertoires of antibody variable domains in which consensus primers directed at or adjacent to the 5' end of the variable domain area are used in conjunction with consensus primers to the third framework region of human VH genes to provide a repertoire of VH variable domains lacking a CDR/CDRs. Marks *et al* further describe how this repertoire may be combined with a CDR of a particular antibody. The repertoire may then be displayed in a suitable host system such as the phage display system of WO92/01047 so that suitable specific binding members may be selected. A repertoire may consist of from anything from 10^4 individual members upwards, for example from 10^6 to 10^8 or 10^{10} members. Analogous shuffling or combinatorial techniques are also disclosed by Stemmer (Nature, 1994, 370:389-391), who describes the technique in relation to a β -lactamase gene but observes that the approach may be used for the generation of antibodies.

[000110] A further alternative is to generate novel VH or VL regions carrying the CDR-derived sequences of the invention using random mutagenesis of, for example, the Ab VH or VL genes to generate mutations within the entire variable domain. Such a technique is described by Gram *et al* (1992, Proc. Natl. Acad. Sci., USA, 89:3576-3580), who used error-prone PCR. Another method which may be used is to direct mutagenesis to CDR regions of VH or VL genes. Such techniques are disclosed by Barbas *et al*, (1994, Proc. Natl. Acad. Sci., USA, 91:3809-3813) and Schier *et al* (1996, J. Mol. Biol. 263:551-567).

[000111] All the above described techniques are known as such in the art and in themselves do not form part of the present invention. The skilled person will be able to use such techniques to provide specific binding members of the invention using routine methodology in the art.

[000112] A substantial portion of an immunoglobulin variable domain will comprise at least the three CDR regions, together with their intervening framework regions. Preferably, the portion will also include at least about 50% of either or both of the first and fourth framework regions, the 50% being the C-terminal 50% of the first framework region and the N-terminal 50% of the fourth framework region. Additional residues at the N-terminal or C-terminal end of the substantial part of the variable domain may be those not normally associated with naturally occurring variable domain regions. For example, construction of specific binding members of the present invention made by recombinant DNA techniques may result in the introduction of N- or C-terminal residues encoded by linkers introduced to facilitate cloning or other manipulation steps. Other manipulation steps include the introduction of linkers to join variable domains of the invention to further protein sequences including immunoglobulin heavy chains, other variable domains (for example in the production of diabodies) or protein labels as provided herein and/or known to those of skill in the art.

[000113] Although in a preferred aspect of the invention specific binding members comprising a pair of binding domains based on sequences substantially set out in Figures 10, 12 and/or 13 are preferred, single binding domains based on either of these sequences form further aspects of the invention. In the case of the binding domains based on the sequence substantially set out in Figure 10, 12 and/or 13, such binding domains may be used as targeting agents for tumor antigens since it is known that immunoglobulin VH domains are capable of binding target antigens in a specific manner. In the case of either of the single chain specific binding domains, these domains may be used to screen for complementary domains capable of forming a two-domain specific binding member which has in vivo properties as good as or equal to the Ab antibody disclosed herein.

[000114] This may be achieved by phage display screening methods using the so-called hierarchical dual combinatorial approach as disclosed in U.S. Patent 5,969,108 in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain specific binding member is selected in accordance with phage display techniques such as those described in that reference. This technique is also disclosed in Marks *et al, ibid*. Phage library and phage display selection systems and techniques are also provided herein.

[000115] Specific binding members of the present invention may further comprise antibody constant regions or parts thereof. For example, specific binding members based on the sequences of Figures 10, 12 and 13 may be attached at their C-terminal end to antibody light chain constant domains including human C κ or C λ chains, preferably C λ chains. Similarly, specific binding members based on the sequences of

Figures 10, 12 or 13 may be attached at their C-terminal end to all or part of an immunoglobulin heavy chain derived from any antibody isotype, *e.g.* IgG, IgA, IgE, IgD and IgM and any of the isotype subclasses, particularly IgG1, IgG2b, and IgG4. IgG1 is preferred.

[000116] The antibodies, or any fragments thereof, may be conjugated or recombinantly fused to any cellular toxin, bacterial or other, *e.g.* pseudomonas exotoxin, ricin, or diphtheria toxin. The part of the toxin used can be the whole toxin, or any particular domain of the toxin. Such antibody-toxin molecules have successfully been used for targeting and therapy of different kinds of cancers, see *e.g.* Pastan, Biochim Biophys Acta. 1997 Oct 24;1333(2):C1-6; Kreitman et al., N Engl J Med. 2001 Jul 26;345(4):241-7; Schnell et al., Leukemia. 2000 Jan;14(1):129-35; Ghetie et al., Mol Biotechnol. 2001 Jul;18(3):251-68.

[000117] Bi- and tri-specific multimers can be formed by association of different scFv molecules and have been designed as cross-linking reagents for T-cell recruitment into tumors (immunotherapy), viral retargeting (gene therapy) and as red blood cell agglutination reagents (immunodiagnostics), see *e.g.* Todorovska et al., J Immunol Methods. 2001 Feb 1;248(1-2):47-66; Tomlinson et al., Methods Enzymol. 2000;326:461-79; McCall et al., J Immunol. 2001 May 15;166(10):6112-7.

[000118] Fully human antibodies can be prepared by immunizing transgenic mice carrying large portions of the human immunoglobulin heavy and light chains. These mice, examples of such mice are the Xenomouse™ (Abgenix, Inc.) (US Patent Nos. 6,075,181 and 6,150,584), the HuMAb-Mouse™ (Medarex, Inc./GenPharm) (US patent 5545806 and 5569825), the TransChromo Mouse™ (Kirin) and the KM Mouse™ (Medarex/Kirin), are well known within the art. Antibodies can then be prepared by, *e.g.* standard hybridoma technique or by phage display. These antibodies will then contain only fully human amino acid sequences. Fully human antibodies can also be generated using phage display from human libraries. Phage display may be performed using methods well known to the skilled artisan, and as provided herein as in Hoogenboom et al and Marks et al (Hoogenboom HR and Winter G. (1992) J Mol Biol. 227(2):381-8; Marks JD et al (1991) J Mol Biol. 222(3):581-97; and also U.S. Patents 5885793 and 5969108).

[000119] Antibodies of the invention may be labelled with a detectable or functional label. Detectable labels include, but are not limited to, radiolabels such as the isotopes ³H, ¹⁴C, ³²P, ³⁵S, ³⁶Cl, ⁵¹Cr, ⁵⁷Co, ⁵⁸Co, ⁵⁹Fe, ⁹⁰Y, ¹²¹I, ¹²⁴I, ¹²⁵I, ¹³¹I, ¹¹¹In, ¹¹⁷Lu, ²¹¹At, ¹⁹⁸Au, ⁶⁷Cu, ²²⁵Ac, ²¹³Bi, ⁹⁹Tc and ¹⁸⁶Re, which may be attached to antibodies of the invention using conventional chemistry known in the art of antibody imaging.

Labels also include fluorescent labels (for example fluorescein, rhodamine, Texas Red) and labels used conventionally in the art for MRI-CT imaging. They also include enzyme labels such as horseradish peroxidase, β -glucuronidase, β -galactosidase, urease. Labels further include chemical moieties such as biotin which may be detected via binding to a specific cognate detectable moiety, *e.g.* labelled avidin. Functional labels include substances which are designed to be targeted to the site of a tumor to cause destruction of tumor tissue. Such functional labels include cytotoxic drugs such as 5-fluorouracil or ricin and enzymes such as bacterial carboxypeptidase or nitroreductase, which are capable of converting prodrugs into active drugs at the site of a tumor.

[000120] Also, antibodies including fragments thereof, and drugs that modulate the production or activity of the specific binding members, antibodies and/or their subunits may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring conditions such as cancer, precancerous lesions, conditions related to or resulting from hyperproliferative cell growth or the like. For example, the specific binding members, antibodies or their subunits may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Likewise, small molecules that mimic or antagonize the activity(ies) of the specific binding members of the invention may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

[000121] The radiolabelled specific binding members, particularly antibodies and fragments thereof, are useful in *in vitro* diagnostics techniques and in *in vivo* radioimaging techniques and in radioimmunotherapy. In the instance of *in vivo* imaging, the specific binding members of the present invention may be conjugated to an imaging agent rather than a radioisotope(s), including but not limited to a magnetic resonance image enhancing agent, wherein for instance an antibody molecule is loaded with a large number of paramagnetic ions through chelating groups. Examples of chelating groups include EDTA, porphyrins, polyamines crown ethers and polyoximes. Examples of paramagnetic ions include gadolinium, iron, manganese, rhenium, europium, lanthanum, holmium and fermium. In a further aspect of the invention, radiolabelled specific binding members, particularly antibodies and fragments thereof, particularly radioimmunoconjugates, are useful in radioimmunotherapy, particularly as radiolabelled antibodies for cancer therapy. In a still further aspect, the radiolabelled specific binding members, particularly antibodies and fragments thereof, are useful in radioimmuno-guided surgery techniques, wherein they can identify and indicate the presence and/or location of cancer cells, precancerous cells, tumor cells, and hyperproliferative cells, prior to, during or following surgery to remove such cells.

[000122] Immunoconjugates or antibody fusion proteins of the present invention, wherein the specific binding members, particularly antibodies and fragments thereof, of the present invention are conjugated or attached to other molecules or agents further include, but are not limited to binding members conjugated to a chemical ablation agent, toxin, immunomodulator, cytokine, cytotoxic agent, chemotherapeutic agent or drug.

[000123] Radioimmunotherapy (RAIT) has entered the clinic and demonstrated efficacy using various antibody immunoconjugates. ¹³¹I labeled humanized anti-carcinoembryonic antigen (anti-CEA) antibody hMN-14 has been evaluated in colorectal cancer (Behr TM et al (2002) Cancer 94(4Suppl):1373-81) and the same antibody with ⁹⁰Y label has been assessed in medullary thyroid carcinoma (Stein R et al (2002) Cancer 94(1):51-61). Radioimmunotherapy using monoclonal antibodies has also been assessed and reported for non-Hodgkin's lymphoma and pancreatic cancer (Goldenberg DM (2001) Crit Rev Oncol Hematol 39(1-2):195-201; Gold DV et al (2001) Crit Rev Oncol Hematol 39 (1-2) 147-54). Radioimmunotherapy methods with particular antibodies are also described in U.S. Patent 6,306,393 and 6,331,175. Radioimmunoguided surgery (RIGS) has also entered the clinic and demonstrated efficacy and usefulness, including using anti-CEA antibodies and antibodies directed against tumor-associated antigens (Kim JC et al (2002) Int J Cancer 97(4):542-7; Schneebaum S et al (2001) World J Surg 25(12):1495-8; Avital S et al (2000) Cancer 89(8):1692-8; McIntosh DG et al (1997) Cancer Biother Radiopharm 12 (4):287-94).

[000124] In vivo animal models of cancer or animal xenograft studies may be utilized by the skilled artisan to further or additionally screen, assess, and/or verify the specific binding members and antibodies or fragments thereof of the present invention, including further assessing NY-ESO-1 modulation and immunotherapy in vivo. Such animal models include, but are not limited to models of melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder cancer and synovial sarcoma.

[000125] Antibodies of the present invention may be administered to a patient in need of treatment via any suitable route, including by injection into the bloodstream or CSF, or directly into the site of the tumor. The precise dose will depend upon a number of factors, including whether the antibody is for diagnosis or for treatment, the size and location of the tumor, the precise nature of the antibody (whether whole antibody, fragment, diabody, etc), and the nature of the detectable or functional label attached to the antibody. Where a radionuclide is used for therapy, a suitable maximum single dose may be about 45 mCi/m², to a maximum of about 250 mCi/m². Preferable dosage is in the range of 15 to 40 mCi, with a

further preferred dosage range of 20 to 30 mCi, or 10 to 30 mCi. Such therapy may require bone marrow or stem cell replacement. A typical antibody dose for either tumor imaging or tumor treatment will be in the range of from 0.5 to 40 mg, preferably from 1 to 4 mg of antibody in F(ab')₂ form. Naked antibodies are preferably administered in doses of 20 to 1000 mg protein per dose, or 20 to 500 mg protein per dose, or 20 to 100 mg protein per dose. This is a dose for a single treatment of an adult patient, which may be proportionally adjusted for children and infants, and also adjusted for other antibody formats, in proportion for example to molecular weight. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician.

Pharmaceutical and Therapeutic Compositions

[000126] Specific binding members of the present invention will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the specific binding member. Thus pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous, or by deposition at a tumor site.

[000127] Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

[000128] For intravenous, injection, or injection at the site of affliction, the active ingredient may be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

[000129] A composition may be administered alone or in combination with other treatments, therapeutics or agents, either simultaneously or sequentially dependent upon the condition to be treated. In addition, the present invention contemplates and includes compositions comprising the binding member, particularly antibody or fragment thereof, herein described and other agents or therapeutics such as anti-cancer agents or therapeutics, hormones, anti-mitotic agents, anti-apoptotic agents, antibodies, or immune modulators. More generally these anti-cancer agents may be but are not limited to tyrosine kinase inhibitors or phosphorylation cascade inhibitors, post-translational modulators, cell growth or division inhibitors (*e.g.* anti-mitotics), or signal transduction inhibitors. Other treatments or therapeutics may include the administration of suitable doses of pain relief drugs such as non-steroidal anti-inflammatory drugs (*e.g.* aspirin, paracetamol, ibuprofen or ketoprofen) or opiates such as morphine, or anti-emetics. The composition can be administered in combination (either sequentially (*i.e.* before or after) or simultaneously) with tyrosine kinase inhibitors (including, but not limited to AG1478 and ZD1839, STI571, OSI-774, SU-6668), doxorubicin, temozolomide, cisplatin, carboplatin, nitrosoureas, procarbazine, vincristine, hydroxyurea, 5-fluoruracil, cytosine arabinoside, cyclophosphamide, epipodophyllotoxin, carmustine, lomustine, and/or other chemotherapeutic agents. Thus, these agents may be specific anti-cancer agents, or immune cell response modulators or may be more general anti-cancer and anti-neoplastic agents such as doxorubicin, cisplatin, temozolomide, nitrosoureas, procarbazine, vincristine, hydroxyurea, 5-fluoruracil, cytosine arabinoside, cyclophosphamide, epipodophyllotoxin, carmustine, or lomustine. In addition, the composition may be administered with hormones such as dexamethasone, immune modulators, such as interleukins, tumor necrosis factor (TNF) or other growth factors, colony stimulating factors, or cytokines which stimulate the immune response and reduction or elimination of cancer cells or tumors. The composition may also be administered with, or may include combinations along with other anti-tumor antigen antibodies.

[000130] In addition, the present invention contemplates and includes therapeutic compositions for the use of the binding member in combination with conventional radiotherapy.

[000131] The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more of a specific binding member, polypeptide analog thereof or fragment thereof, as described herein as an active ingredient. In a preferred embodiment, the composition comprises an antigen capable of modulating the specific binding of the present binding member/antibody with a target cell.

[000132] The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions. However, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

[000133] A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[000134] The therapeutic antibody- or active fragment-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; *i.e.*, carrier, or vehicle.

[000135] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of peptide/MHC or tumor antigen binding capacity desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. Suitable regimes for initial administration and follow on administration are also variable, and may include an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain appropriate and sufficient concentrations in the blood or at the site of desired therapy are contemplated.

[000136] Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

[000137] For intravenous, injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Diagnostic Assays

[000138] The present invention also relates to a variety of diagnostic applications, including methods for detecting the presence of NY-ESO-1/MHC complexes, NY-ESO-1 antigen, NY-ESO-1 mediated cancer, or cancer more generally, by reference to their ability to be recognized by the present specific binding member(s). Peptide complexes can be identified, targeted, labeled, and/or quantitated on dendritic cells or other antigen presenting cells and/or tumor cells.

[000139] Diagnostic applications of the specific binding members of the present invention, particularly antibodies and fragments thereof, include *in vitro* and *in vivo* applications well known and standard to the skilled artisan and based on the present description. Diagnostic assays and kits for *in vitro* assessment and evaluation of tumor and cancer status, may be utilized to diagnose, evaluate and monitor patient samples including those known to have or suspected of having cancer, a precancerous condition, a condition related to hyperproliferative cell growth or from a tumor sample. The assessment and evaluation of cancer, tumor and metastatic disease status is also useful in determining the suitability of a patient for a clinical trial of a drug or for the administration of a particular chemotherapeutic agent or specific binding member, particularly an antibody, of the present invention, including combinations thereof, versus a different agent or binding member. This type of diagnostic monitoring and assessment is already in practice utilizing antibodies against the HER2 protein in breast cancer (Hercep Test, Dako Corporation), where the assay is also used to evaluate patients for antibody therapy using Herceptin. *In vivo* applications include imaging of tumors or assessing cancer status of individuals, including radioimaging.

[000140] Preferably, the antibody used in the diagnostic methods of this invention is human antibody. More preferably, the antibody is a single chain antibody or domain antibody. In addition, the antibody molecules used herein can be in the form of Fab, Fab', F(ab')₂ or F(v) portions of whole antibody molecules, particularly Fab.

[000141] As described in detail above, antibody(ies) to the NY-ESO-1/MHC molecule complex(es) can be produced and isolated by standard methods including the phage display techniques and mutagenesis and recombinant techniques.

[000142] The presence of tumor antigen peptide/MHC complex(es), particularly of NY-ESO-1/MHC complexes, in cells can be ascertained by the usual *in vitro* or *in vivo* immunological procedures applicable to such determinations. A number of useful procedures are known. The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. The "competitive" procedure is described in U.S. Patent Nos. 3,654,090 and 3,850,752. The "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and 4,016,043. Still other procedures are known such as the "double antibody," or "DASP" procedure.

[000143] In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of aberrant expression of including but not limited to amplified and/or an mutation, in suspected target cells. In accordance with the testing techniques discussed above, one class of such kits will contain at least the labeled or its binding partner, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

[000144] Accordingly, a test kit may be prepared for the demonstration of the presence of tumor antigen peptide/MHC complexes, comprising:

(a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of the present specific binding member or a specific binding partner thereto, to a detectable label;

(b) other reagents; and

(c) directions for use of said kit.

[000145] A test kit may be prepared for the demonstration of the presence of cancer, particularly selected from melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder cancer, and synovial sarcoma, comprising:

- (a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of the present specific binding member or a specific binding partner thereto, to a detectable label;
- (b) other reagents; and
- (c) directions for use of said kit.

[000146] In accordance with the above, an assay system for screening potential drugs effective to modulate the presence or activity of the tumor antigen peptide/MHC complex and/or the activity or binding of the antibody of the present invention may be prepared. The antigen peptide or the binding member or antibody may be introduced into a test system, and the prospective drug may also be introduced into the resulting cell culture, and the culture thereafter examined to observe any changes in the activity of the cells, binding of the antibody, or amount and extent of tumor antigen peptide/MHC complex due either to the addition of the prospective drug alone, or due to the effect of added quantities of the known agent(s).

Nucleic Acids

[000147] The present invention further provides an isolated nucleic acid encoding a specific binding member of the present invention. Nucleic acid includes DNA and RNA. In a preferred aspect, the present invention provides a nucleic acid which codes for a polypeptide of the invention as defined above, including a polypeptide as set out in Figures 10, 12 or 13 or capable of encoding the CDR regions thereof. The invention provides exemplary nucleic acid of Figure 11 which encodes the amino acid of Figure 10.

[000148] The present invention also provides constructs in the form of plasmids, vectors, transcription or expression cassettes which comprise at least one polynucleotide as above. The present invention also provides a recombinant host cell which comprises one or more constructs as above. A nucleic acid encoding any specific binding member as provided itself forms an aspect of the present invention, as does a method of production of the specific binding member which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression a specific binding member may be isolated and/or purified using any suitable technique, then used as appropriate.

[000149] Specific binding members and encoding nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes origin other than the sequence encoding a polypeptide with the required function. Nucleic acid according to the present invention may comprise DNA or RNA and may be wholly or partially synthetic.

[000150] Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is *E.coli*. The expression of antibodies and antibody fragments in prokaryotic cells such as *E.coli* is well established in the art.

[000151] Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Short Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

[000152] Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene. The present

invention also provides a method which comprises using a construct as stated above in an expression system in order to express a specific binding member or polypeptide as above.

[000153] Another feature of this invention is the expression of the DNA sequences disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host. A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col E1, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage λ , e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2u plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

[000154] Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the *LTR* system, the major operator and promoter regions of phage λ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast α -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[000155] A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, YB/20, NSO, SP2/0, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture.

[000156] It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function

equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, *e.g.*, their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products. Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

[000157] As mentioned above, a DNA sequence encoding a specific binding member can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the specific binding member amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, *e.g.*, Edge, *Nature*, **292**:756 (1981); Nambair et al., *Science*, **223**:1299 (1984); Jay et al., *J. Biol. Chem.*, 259:6311 (1984). Synthetic DNA sequences allow convenient construction of genes which will express specific binding member analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native specific binding member genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis.

[000158] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

Rational Development of High Affinity T-Cell Receptor-Like Antibodies

[000159] T-cell interaction with a target cell is a key event in adaptive immune response and primarily driven by T-cell receptor (TCR) recognition of peptide-MHC (pMHC) complexes. TCR avidity for a given pMHC is determined by number of MHC molecules, availability of co-receptors and TCR affinity for MHC or peptide, respectively, with peptide recognition being the most important factor to confer target specificity. Here we present high resolution crystal structures for two Fab antibodies in complex with the immunodominant NY-ESO-1₁₅₇₋₁₆₅ peptide analogue (SLLMWITQV) presented by HLA-A*0201 and compare them against a TCR recognizing the same pMHC. Both, binding to the central methionine-tryptophan (MW) peptide motif and orientation of binding were almost identical for Fabs and TCR. As the MW 'peg' dominates the contacts between Fab and peptide, we estimated the contributions of individual amino acids between the Fab and peptide to provide the rational basis for a peptide focused second-generation, high affinity antibody library. The final Fab candidate achieved better peptide binding by two light chain mutations giving a twenty-fold affinity improvement to 2-4 nM, exceeding the affinity of the TCR by 1000-fold. The high affinity Fab when grafted as recombinant TCR on T-cells conferred specific killing of HLA-A*0201/ NY-ESO-1₁₅₇₋₁₆₅ target cells. In summary, we prove that affinity maturation of antibodies mimicking a TCR is possible and provide a strategy for engineering of high affinity antibodies that can be used in targeting specific pMHC complexes for diagnostic and therapeutic purposes.

Introduction

[000160] The complex formed by the Major-Histocompatibility-Complex (MHC) class I molecule and a peptide primarily derived from a cytoplasmic protein (pMHC) represents a unique, cell surface exposed marker (1). T-cell receptors (TCRs) as natural ligands of pMHC complexes can target the pMHC efficiently (2) but generally have low binding affinities preventing their use as therapeutic reagents (3). Therefore, high affinity recombinant TCRs would be ideal candidates for diagnostic and even therapeutic purposes (4). Indeed, affinity maturation strategies have been successfully developed and demonstrated dramatic increases of TCR affinity (5, 6). However, random mutagenesis was sometimes hampered by loss of peptide specificity resulting in cross-reactivity with control pMHC complexes. Gain of affinity was the direct consequence of tighter binding to the MHC backbone (7) indicating that the knowledge of the precise TCR-pMHC interaction could be critical for the development of specific, high affinity TCRs.

[000161] We previously determined the structure of the NY-ESO-1₁₅₇₋₁₆₅/HLA-A*0201 complex recognised by a specific TCR (1G4) with moderate affinity of 3-5 μ M (8). Whilst the 1G4 TCR binds in a classical diagonal orientation with respect to the peptide axis, it notably centres on the peptide's hydrophobic prominently exposed methionine (M) and tryptophan (W) side chains at residues 4 and 5 of the NY-ESO-1₁₅₇₋₁₆₅ peptide. This MW motif is enveloped by the TCR's CDR loops and contributes approximately 50% of the buried surface area contacted between TCR and peptide. Both M and W are encoded by single codons each, making the combination of these amino acids rare in the human proteome and an uncommon motif found in peptides presented by MHC molecules. In order to develop a reagent with diagnostic or even therapeutic potential, random mutagenesis of the 1G4 TCR was performed and resulted in an impressive 220,000 fold increase of binding affinity (9). However, tighter binding was again associated with loss of peptide specificity when tested in a cellular assay (4). Therefore, new approaches replacing the TCR as a binding tool have to be established. One option could be the use of antibodies since they share structural homology with TCRs regarding target recognition and usually confer higher affinity with excellent specificity (10).

[000162] Indeed, phage display libraries have recently enabled the rapid isolation of human Fab fragments highly specific to pMHC molecules (11). The structure of one Fab fragment (Hyb3) bound to the HLA-A*1-MAGE-A1 peptide complex has been solved demonstrating considerable deviation from a normal TCR-like binding footprint (12) although, detailed comparison of Hyb3 and HLA-A*1-MAGE-A1 specific TCR binding modes was precluded because of the lack of a representative TCR-pMHC structure. Hyb3 was generated by random affinity maturation raising the question that the recognition focus on the MHC alpha-1 (α 1) helix, rather than peptide, might be the result of this process. T-cells grafted with Hyb3 as recombinant TCR had lost peptide specificity and killed HLA-A*1 positive cells irrespective of peptide presentation (13). Therefore, shifting the binding affinity from the MHC helices to the peptide and keeping peptide specificity is the critical issue even in antibody based approaches when aiming for high affinity pMHC binders. Here we describe a two step procedure where we first compared the high resolution structure of two HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ specific Fab antibodies (3M4E5 and 3M4F4 Fab) bound to the complex with the structure of the corresponding 1G4 TCR. In a second step, these structural data were used to generate a new antibody library on the basis of Fab 3M4E5. Key residues making contact to the MW motif were conserved whilst others were randomised where individual side chains could be optimised to interact with the peptide but not with the MHC backbone. To our knowledge, this is the first time that a rational strategy like this has been successfully followed for the affinity maturation of a T-cell receptor like antibody.

[000163] Data deposition: Atomic coordinates and structure factors for the proteins and complexes have been deposited with the Protein Data Bank under the following accession codes: HLA-A2-NYESO-1-3M4E5, XXX; HLA-A2-NYESO-1-3M4F4, XXX; and 3M4E5 Fab, XXX.

Results

[000164] TCR-like binding properties of phage-derived Fabs to HLA-A*0201-NY-ESO-1 complexes. The distinctive, exposed MW motif of NY-ESO-1₁₅₇₋₁₆₅ antigen (Fig. 1*A* and *B*) may facilitate the generation of suitably peptide focused Fabs. We had selected from a naïve antibody phage library (14) NY-ESO-1₁₅₇₋₁₆₅/HLA-A*0201 specific Fabs (3M4E5 and 3M4F4) with 100-fold higher affinities than the soluble 1G4 TCR, principally from much slower Koff rates (TABLE 1). The sequences of both Fabs were very similar with identical CDR3 VH and CDR3 VL loops and only a small number of relatively conservative amino acid differences in the CDR1 VH, CDR2 VH and CDR2 VL loop regions. Crystal structures of the Fab-pMHC complexes and Fab 3M4E5 in isolation at 1.9Å, 2.9Å and 2.3Å resolution (TABLE 2) revealed 1G4 TCR-like binding modes. The VH and VL domains of the bound Fabs broadly superposed with the 1G4 TCR Vα and Vβ domains (Fig. 1*A*, *C* and *D*), respectively, with diagonal binding angles of 69° for the 1G4 TCR (8) and 40° for the Fab 3M4E5. Like the TCR, the Fabs focused the central ‘hotspot’ of their binding footprint onto the MW peg with the CDR2 VH, CDR3 VH and CDR3 VL loops playing similar roles to the equivalent TCR Vα/Vβ loops (FIGURE 1*A* and 1*D*; TABLE 3). In particular, TCR CDR3α residue Y100 and Fab VH CDR3 residue Y103 made hydrogen bonds to the peptide main chain carbonyl oxygen of M4 and their aromatic tyrosine rings interacted closely with the hydrophobic MW motif (FIGURE 2). Additionally, in both TCR and Fabs, the hydrophobic portion of a large residue (TCR R93α and Fab Y98_{VL}), arched over the MW motif and was stabilized through hydrogen bonds to the counterpart CDR3 loop (TCR S93β and Fab E99_{VH}) (FIGURE 1*E* and 1*F*).

TABLE 1

SPR affinity measurements for TCR 1G4 and Fabs 3M4E5, 3M4F4 and Fab T1.
 NY-ESO-1₁₅₇₋₁₆₅ peptides carried a cysteine (9C) or valine(9V) at position 9.

Analyte	A2-NY-ESO-1 peptide	ka (mol-1.s-1)	kd (s-1)	KD (nM)
1G4 TCR	9C	87500	0.357	4080
	9V	53425	0.078	1460
3M4E5	9C	93700	0.004225	47
	9V	80850	0.00362	46
3M4F4	9C	68800	0.00651	95
	9V	73700	0.00467	63
Fab T1	9C	138375	0.000674	2
	9V	249000	0.000835	4

TABLE 2
Crystallographic data collection and refinement statistics.

Data Collection	3M4E5-A2-NYESO-1 (1.9Å)	3M4F4-A2-NYESO-1 (2.9Å)	2M4E5 (2.3Å)
Space group	P2 ₁	P2 ₁	P4 ₂
Unit cell			
Dimensions (Å) (a, b, c,)	71.6, 111.8, 124.7	70.8, 105.6, 256.5	117.1, 117.1, 78.9
Angles (°) (α,β,γ)	90, 93.5, 90	90, 92.3, 90	90, 90, 90
Molecules/complexes in AU	2	4	2
Source	ESRF ID14-eh2	ESRF ID14-eh2	ESRF ID14-eh2
Resolution (Å)			
(highest resolution shell)	30 – 1.9 (1.97 – 1.90)*	30 – 2.9 (2.70 – 2.9)*	30 – 2.3 (2.45 – 2.3)*
Measured reflections	977096	539019	428360
Unique reflections	153950	84609	24603
Completeness (%)	100 (99.9)*	97.8 (88.5)*	99.0 (92.1)*
I/σ (I)	23.0 (2.6)*	14.2 (2.6)*	40.9 (4.4)*
R _{merge} (%) ^a	7.2 (72.7)*	11.8 (52.3)*	6.5 (63.1)*
Refinement Statistics			
Resolution range (Å)	30 – 1.9 (1.97 – 1.90)*	30 – 2.9 (3.0 – 2.9)*	30 – 2.3 (2.45 – 2.3)*
R _{cryst} ^b	20.4	20.5	22.2
R _{free} ^c	25.1	28.2	30.4
Number of non-H protein atoms	12715	25434	6412
Number of water molecules	1606	0	379
Rms deviation from ideality			
Bond lengths (Å)	0.014	0.017	0.021
Bond angles (°)	1.62	1.82	1.946
Ramachandran Plot (%) (favored, (84.7, 13.6, 1.1, 0.6) allowed, generous, disallowed)		(86.0, 12.1, 1.2, 0.7)	(85.1, 13.0, 1.3, 0.7)

^a $R_{merge} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$ where I is the intensity of unique reflection hkl and $\langle I \rangle$ is the average over symmetry-related observations of unique reflection hkl .

^b $R_{cryst} = \sum |F_{obs} - F_{calc}| / \sum F_{obs}$ where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.

^c R_{free} is calculated as for R_{cryst} but using 5.0% of reflections sequestered before refinement.

* numbers in parentheses correspond to the outermost shell of data.

[000165] The Fabs and 1G4 TCR shared many contact residues with HLA-A*0201 (R65, K66, A69, Q72, T73, A150, and Q155), including hydrogen bonds to the same atoms (R65 NE, T73 OG, A150 O and Q155 E1). Fab 3M4E5 had significantly lower protein-protein shape complementarity to the peptide ($Sc = 0.85$ with waters, 0.74 without waters) compared to that of the 1G4 TCR interface ($Sc = 0.87$ with waters, 0.81 without waters), and Fab 3M4E5 trapped more water molecules at the interface, notably 4 located above the hydrophobic MW motif (FIGURE 1C and 1D). Multiple copies of the Fab-pMHC complexes in the crystallographic asymmetric unit when superposed revealed some 'rocking' in relative orientation of Fab and pMHC is permitted (FIGURE 3A). We and others have observed similar evidence of flexion in

TCR-pMHC complexes (15, 16). Comparison of the crystal structures of liganded and unliganded 3M4E5 Fab revealed only very small changes in the antigen combining site (FIGURE 4), with the largest change being where the Q32_{HC} side chain flips 180° to make space for the MW motif. However, comparison of the 4 3M4F4 Fab-HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complexes revealed two VH CDR2 conformations: an 'upward' and a 'downward' position, differing by a maximum of 6.7Å in the main chain (FIGURE 1B, FIGURES 3B and 3C).

[000166] Selecting amino acid positions for affinity maturation. Since the NY-ESO-1₁₅₇₋₁₆₅/HLA-A*0201 specific Fabs 3M4E5 and 3M4F4 were of moderate affinity (TABLE 1) compared to therapeutic antibodies used in the clinic, we aimed to affinity mature the Fab 3M4E5 based on our structural data. Previous studies had indicated that the 'hotspot' of the interaction to a protein antigen remained conserved during affinity maturation (17). Gains in binding energy could be achieved by (i) stabilising the bound conformation of any flexible CDR loops in the unliganded Fab and (ii) optimising interactions in the periphery of the binding interface (i.e. around the 'hotspot'). (i) looked a less promising route to affinity maturation since the structural data indicated a rather limited induced fit on binding of Fab 3M4E5 to the NY-ESO-1₁₅₇₋₁₆₅/HLA-A*0201 complex. Therefore, we focussed on the interacting amino acids around the 'hotspot' MW motif and residues directly interacting with the MW motif were held invariant. We randomized amino acids at positions where side chains could be optimized for direct peptide interaction (FIGURE 5A-D; TABLE 3B) to enhance Fab affinity whilst retaining peptide specificity. Residues orientated away from the peptide or towards the MHC helices were not included to avoid ineffective changes or unwanted increase of affinity to the MHC helices. For the light chain, the structural data suggested residues 26S, 27R, 32Y, 95G, 96S and 97Y as candidates for variation.

TABLE 3A and 3B

(A) Interactions between 1G4 TCR V α and V β domains and the HLA-A*0201- NY-ESO-1₁₅₇₋₁₆₅ (<4.0Å) and

(B) Interactions between Fab 3M4E5 VL and VH domains and the HLA-A*0201- NY-ESO-1₁₅₇₋₁₆₅ (<4.0Å)

Table 3A

Direct interactions (<4.0Å)		
Element	1G4 TCR contact residue	HLA-A2 contact residue
V α CDR1	Y31	Q155
V α CDR2	Q51	A150, H151, Q155
V α CDR2	S52	Q155
V α CDR2	S53	Q155, E154
V α CDR2	S54	H151, E154
V α CDR3	G97	R65
V α CDR3	G98	R65, K66
V α CDR3	S99	R65
V α CDR3	Y100	K66
V β CDR1	E29	Q72, T73
V β CDR2	Y47	R65
V β CDR2	V49	R65, Q72
V β CDR2	G50	Q72
V β CDR2	A51	Q72
V β CDR2	I53	K68
V β CDR2	D55	R65
V β CDR2	T70	Q72
V β CDR3	V95	T73
V β CDR3	N97	A150
Element	1G4 TCR contact residue	Peptide contact residue
V α CDR1	Y31	W5
V α CDR3	R93	W5
V α CDR3	P94	M4, W5
V α CDR3	T95	M4, W5
V α CDR3	S96	M4
V α CDR3	G97	M4
V α CDR3	G98	M4
V α CDR3	Y100	M4
V β CDR1	N27	Q8
V β CDR1	E29	Q8
V β CDR3	Y94	Q8
V β CDR3	V95	M4, W5, I6
V β CDR3	G96	W5, I6, T7
V β CDR3	N97	T7

Table 3B

Direct interactions (<4.0Å)		
Element	3M4E5 contact residue	HLA-A2 contact residue
VH CDR2	V52	T163
VH CDR2	S54	E166, T167
VH CDR2	G55	T163
VH CDR2	G56	A158, T163
VH CDR2	S57	T163, Y159
VH CDR2	T58	A158, Q155
VH CDR2	A59	Q155
VH CDR2	K65	A150, H152
VH CDR3	L101	R65
VH CDR3	P102	R65
VH CDR3	Y103	R65, K66, A69
VH CDR3	Y104	R65
VL CDR1	G31	Q72
VL CDR1	Y32	Q72, A69, T73
VL CDR1	Y34	R65
VL CDR2	D52	R65
VL CDR3	S96	Q155
Element	3M4E5 contact residue	Peptide contact residue
VH CDR2	S57	W5
VH CDR2	T58	W5
VH CDR2	A59	W5
VH CDR3	P102	M4
VH CDR3	Y103	M4
VL CDR1	S26	Q8
VL CDR1	R27	Q8
VL CDR1	Y32	Q8
VL CDR3	F93	M4, W5
VL CDR3	G95	W5, I6
VL CDR3	S96	W5, T7
VL CDR3	Y97	W5
VL CDR3	Y98	M4, W5

[000167] Residue identification for the heavy chain was more complicated as a less clear cut discrimination between residues interacting with peptide and with HLA-A*0201 could be established (FIGURE 5A-D; TABLE 3B). In the Fab 3M4E5 complex structure, the large water-filled cavity within the MW binding pocket, occupied by 4 water molecules, was surrounded by residues Q33_{VH}, S35_{VH}, G50_{VH} and E99_{VH} (FIGURE 1D; FIGURE 5B, 5C and 5D). The higher B factors for these waters (average 37Å², n=4) compared to other water molecules trapped at the interface (average 30.5Å², n=8) suggested that these waters were not stably positioned within the interface, and thus side chain alterations in this pocket could stabilize the association of the Fab to the peptide. In addition, positions V52_{VH}, S57_{VH} and A59_{VH} were identified as candidate positions to enhance affinity by changing the conformational stability of the CDR2 loop inferred from the 3M4F4 Fab-HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complex.

[000168] Identifying higher affinity Fabs from the 2nd generation library. The final antibody library contained 10⁸ independent clones and after the 3rd round of selection, 480 candidate clones were identified of which 172 revealed, as soluble phage particles, specific binding to the HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complex. Sequence data of strong binders were grouped by cluster analysis and three types (Fab T1-3) of repeatedly selected mutants could be identified by phylogenetic tree analysis (FIGURE 6). The most dominant mutations present in all three types of mutants included S26E (LC CDR1) and S96G (LC CDR3), respectively. Fab T1 contained only these amino acid (AA) changes, whereas Fab T2 and T3 had additional heavy chain mutations (TABLE 4). The amino acid sequences of the heavy chain and light chain variable regions of the T1, T2 and T3 antibodies are shown in Figures 10, 12 and 13 respectively, with the CDR regions depicted in blue.

TABLE 4

Listing of the 3 most frequent sequence patterns selected from the 2nd generation phage display library. Only residues that were randomised as selection strategy are depicted. Amino acid positions are given as Kabat numbers (<http://www.hgmp.mrc.ac.uk>)

	Light chain						Heavy chain						frequency of clones	
Amino acid position	26	27	32	95	96	97	33	35	50	52	57	59	99	
3M4E5	S	R	Y	G	S	Y	Q	S	G	V	S	A	E	
Fab T1	<u>E</u>	R	Y	G	<u>G</u>	Y	Q	S	G	V	S	A	E	n = 15
Fab T2	<u>E</u>	R	Y	G	<u>G</u>	Y	<u>E</u>	<u>H</u>	<u>V</u>	<u>L</u>	<u>E</u>	<u>N</u>	<u>M</u>	n = 11
Fab T3	<u>E</u>	R	Y	G	<u>G</u>	Y	<u>Y</u>	<u>D</u>	<u>T</u>	<u>A</u>	<u>E</u>	A	<u>S</u>	n = 11

[000169] All three Fab candidates demonstrated an equivalent or even stronger binding signal by ELISA on NY-ESO-1₁₅₇₋₁₆₅/HLA-A*0201 complexes at different concentrations when compared to Fab 3M4E5 (FIGURE 7A). The strongest signal was detected for Fab T1. All three Fab candidates (T1-T3) conserved the precise binding specificity of Fab 3M4E5 and did not cross-react to other HLA-A*0201 complexes displaying an irrelevant peptide (data not shown). Moreover, Fabs T1-T3 reacted only with HLA-A*0201⁺ T2 cells presenting the NY-ESO-1₁₅₇₋₁₆₅ peptide but not any partially overlapping NY-ESO-1 peptide sequences (FIGURE 7B). Inhibition experiments on NY-ESO-1₁₅₇₋₁₆₅ peptide pulsed T2 cells (FIGURE 7C) using non-saturating amounts of biotinylated 3M4E5 tetramer indicated differences in

binding affinities for T1-T3. Fab T1 demonstrated the highest binding affinity by achieving a significantly stronger inhibitory signal when compared to Fab 3M4E5 or Fab T2 and T3, respectively. The same pattern of inhibition was observed in a cellular assay with the strongest T-cell inhibition capacity, for Fab T1, significantly exceeding the parental Fab 3M4E5 (FIGURE 7D). These functional data were in line with affinity and kinetic measurements demonstrating a 20 fold affinity improvement for Fab T1, achieving 2-4 nM affinities as anticipated (TABLE 1).

[000170] Translating affinity into increased T-cell activity. Recombinant single chain TCRs were generated to demonstrate the biological relevance of Fab 3M4E5 and T1 in T-cell based assays. For that purpose, CD3⁺ T-cells were isolated from the peripheral blood of healthy donors and retrovirally transduced *in-vitro* with single-chain (sc)-3M4E5 TCR, sc-T1 TCR or sc-anti-CEA TCR as control (18). Transduction efficacy varied between 40-50% and correct functional assembly of immune-receptors was confirmed by HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ tetramer staining demonstrating adequate expression levels for sc-3M4E5 TCR and sc-T1 TCR, respectively (FIGURE 8). Sc-3M4E5 TCR and sc-T1 TCR grafted T-cells specifically recognised and killed HLA-A*0201⁺ T2 cells (FIGURE 9) presenting the NY-ESO-1₁₅₇₋₁₆₅ peptide but not any HLA-A*0201⁺ T2 cells loaded with Flu peptide (14). Sc-T1 TCR revealed a significantly higher level of IFN- γ release (data not shown) and cytotoxicity (FIGURE 9A and 9B) when compared to the sc-3M4E5 TCR. In addition, both receptors did not loose peptide specificity and killed in a pMHC-restricted fashion.

Discussion

[000171] We describe the structures of two Fabs recognizing the HLA-A*0201-NY-ESO-1 complex to determine the structural basis of their specificity to the NY-ESO-1 peptide, and examine how the structures can assist in generating high-affinity Fab variants keeping their specificity.

[000172] The two Fab complex structures bind in a remarkably similar way compared to the 1G4 TCR. By centering on the peptide MW motif, the Fab is positioned similarly to the TCR, and adopts a diagonal binding orientation that generates very similar MHC contact points on the helices. The structures contrast with the HLA-A*01-MAGE-A1-Hyb3 complex which binds more to the MHC α 1 helix, and samples a smaller portion of the peptide (12). The constraints on MHC class I binding for Fabs derived from phage libraries seem therefore less stringent than for TCRs, although the 3M4E5 and 3M4F4 Fabs adopt similar docking positions as the HLA-A*0201-NY-ESO-1 specific 1G4 TCR. The diagonal orientation of all TCRs and the 3M4E5 and 3M4F4 Fabs appear to sample the peptide at an orientation that

positions the receptors into a 'saddle' in the MHC helices. At this diagonal orientation, much of the peptide can be sampled, particularly the central region, whilst the MHC helices stabilize the interaction through hydrogen bonds, salt bridges and Van der Waal's bonds (TABLE 3B). The rocking motion on pMHC observed with these Fabs and indeed for some TCRs (JM22 and SB27) (16, 19, 20), is generally in line with the axis of the 'saddle', highlighting the axis of flexibility on the pMHC surface that demarcates the two lower regions of the $\alpha 1$ and $\alpha 2$ helices (FIGURE 3A). These data suggest that there are structural constraints that preferentially define a diagonal binding orientation found in most pMHC-receptor complexes, but does not exclude the possibility that CD8/CD4/CD3 co-receptors may define the conserved V α /V β position and the general TCR binding position.

[000173] The NY-ESO-1 peptide in HLA-A*0201 is a relatively unique epitope, in that it presents the M and W side chains for recognition by incoming receptors (8). These side chains form a much larger available binding surface area than conventional HLA-A*0201 peptides, where often one bulky residue forms a key central motif exploited for functional recognition. The large MW motif, therefore, assists in generating peptide-specific interactions, and is an excellent focal feature against which to develop therapeutic Fabs with high specificity and affinity. Since the antibodies selected from the primary phage selection process resulted in good specificity with moderate affinity (14), the footprint of TCR and Fab binding to the MHC backbone and the NY-ESO-1₁₅₇₋₁₆₅ peptide directed the design of the second generation library as described before. Our strategy of keeping key MW motif contact residues conserved whilst randomizing only individual side chains interacting with the peptide but not with the MHC backbone was proven experimentally to be correct. The final affinity matured antibody achieved its 20 fold improved affinity by just two light chain mutations. The double mutation S26E and S96G demonstrated a clear route forward for light chain optimisation since it allowed the residues of the VL CDR1 and CDR3 loops to optimise interactions with the I6, T7 and Q8 peptide residues in accordance with the principle of enhancing affinity through improved binding peripheral to the 'hotspot' (17). Interestingly, the usage of residues in the second generation Fab for this region of the interface appeared to have moved towards that of the TCR – which has greater interaction with this C terminal region of the peptide than that of the original Fab 3M4E5. The most frequently identified heavy chain sequence resulting in highest binding affinity was identical to the original Fab 3M4E5 in amino acid sequence. This might be a result of the underlying nature of the antibody library used for the initial selection of Fab 3M4E5. The library is based on the human germline V_H3-23 fragment with synthetic CDR1 and CDR2 domains exceeding the natural diversity of germline V_H and of immunoglobulin sequences found in mature B-cells (21). Therefore, V_H positions identified in our model to be of relevance for antigen binding had already been under high selection pressure in the selection process of Fab 3M4E5 and could not be further optimised.

[000174] Our data are in line with previous attempts on affinity maturation of TCRs, especially those focusing on the HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complex (22). Random affinity maturation of multiple amino acid positions within the 1G4 TCR resulted in dramatic increase of affinity but loss of specificity (4, 9). The affinity increase was primarily due to tighter binding of the MHC backbone and not peptide-focused. As a consequence, the same group generated 1G4 TCR variants with single or dual amino acid substitutions to selectively improve peptide binding (23). A relatively modest increase in TCR affinity to 280 nM was achieved while maintaining antigen specific reactivity of CD8⁺ T cells. We confirm these data since affinity maturation by replacement of two amino acids in the light did not change peptide specificity when analysed in T-cell assays. Both, Fab 3M4E5 and T1 grafted as recombinant scTCR on T-cells kept their pMHC specificity and the higher affinity of Fab T1 translated into a significantly enhanced cytotoxic activity.

[000175] In conclusion, we demonstrate that pMHC specific Fabs can mimic the binding mode of a TCR and these structural data can be used to generate by rational design antibody variants with single digit nano-molar binding affinity keeping the specificity unchanged. NY-ESO-1 in particular is an attractive target antigen since its expression pattern is restricted to germ cells (lacking MHC class I molecules) and a wide variety of hematological and solid organ tumors (24). However, it is expressed in the nucleus and cytoplasm and, therefore, not accessible for most targeted approaches (25). In contrast, NY-ESO-1 derived peptides such as the NY-ESO-1₁₅₇₋₁₆₅ peptide described here are unique cell surface markers when expressed in the appropriate MHC context (14). The rational, peptide focused approach as described resulted in Fab fragments with low nanomolar affinities and will hopefully be a model for the generation of pMHC specific diagnostic and therapeutic reagents.

Material and Methods

[000176] **MHC-peptide monomers.** The HLA-A*0201 heavy chain with a C-terminal biotinylation tag, β 2m, and the respective HLA-A*0201-restricted peptides were refolded by dilution as previously described (14, 26). For crystallography, HLA-A*0201 heavy chain without the biotinylation tag was used for refolding, and purified as described (27).

[000177] **Antibody crystallisation and data collection.** Nanolitre-scale, sitting drop (100 nl protein solution and 100 nl reservoir solution), vapour-diffusion crystallisation trials were set up in 96-well plates using Cartesian Technologies pipetting robots (28). Single 3M4E5-A2-NYESO-1 and 3M4F4-A2-

NYESO-1 complex crystals grew from stoichiometric mixtures (final concentration 10mg/ml) of purified Fab and HLA-A*0201 proteins at 21°C. The 3M4E5-A2-NYESO-1 complex crystallised in 14% PEG 8000 50mM MES pH 6.5 with crystal dimensions of approximately 110µm by 60µm by 30µm. The 3M4F4-A2-NYESO-1 complex crystallised in 12% PEG 8000 50mM MES pH 6.9 with crystal dimensions of approximately 50µm by 35µm by 10µm. The 3M4E5 Fab crystallised in 14% PEG 8000 50mM MES pH 6.5 with crystal dimensions of about 50µm by 40µm by 20µm. Crystals were harvested and briefly soaked sequentially in reservoir solutions containing 10% and 20% glycerol, then flash-cooled and maintained at 100K in a cryostream (Oxford Cryosystems). Datasets were collected at station ID14eh2 of the ESRF (European Synchrotron Radiation Facility) using an ADSC-Q4 (Area Detector Systems Corporation, San Diego) charged-coupled device (CCD) detector. Datasets were auto-indexed and integrated with the program DENZO followed by scaling with the program SCALEPACK (29). The results are summarized in TABLE 3.

[000178] Structure determination and refinement. All structures were determined by molecular replacement in PHASER (30) using the peptide-MHC class I molecule from the HLA-A*0201-NYESO-1-1G4 TCR complex (PDB accession code 2BNQ) and the Fab fragment from PDB entry 1RZF as search coordinates. Initial rigid body refinement of individual domains ($\alpha 1\alpha 2$, $\alpha 3$, $\beta 2M$, peptide, VH, VL, CH and CL) followed by restrained TLS refinement was performed in REFMAC5 (31). Manual rebuilding was carried out in COOT (32) and water picking in the final stages of refinement was performed with ARPw/ARP (33). All regions of the Fabs and HLA-A*0201-NY-ESO-1 were included in the final models except for residues 154-159 from the light chain (chain L) of the 3M4E5-HLA-A*0201-NYESO-1 complex where no electron density was detectable. Crystallographic statistics for the final models are given in TABLE 3. Figures were generated in CCP4MG (34).

[000179] Phage display selection. For the library construction, an antibody phagemid library was generated by gene-synthesis (GENART, Regensburg, Germany) including random codon mutations (to NNB) at indicated positions and subcloned into the pCES vector. Phage Fab particles were produced and incubated with HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ complex in the presence of Fab 3M4E5 protein (100µg/ml) following the selection procedure as previously described (14). Bound phages were eluted with 100 mM Triethylamin and neutralized with Tris-HCl pH 7.2. Phages were used to infect *E. coli* strain TGI (30 min, 37°C) and bacteria were grown on agar (ON, 30°C). Analysis of genetic distance for individual clones was performed using the cluster-analysis program ClustalW (European Bioinformatics Institute, <http://www.ebi.ac.uk/clustalw/>).

[000180] Antibody binding assays. The specificity of individual phage clones and soluble Fab antibodies was assessed by ELISA at RT with indirectly coated MHC-class I antigen-peptide complexes (14). Correct folding of complexes was confirmed by monoclonal antibodies W6/32 and BB7.2 (Jackson, West Grove, USA). Recombinant soluble Fab antibodies were purified from *E. coli* periplasmic fraction as described (14) and bound Fab-molecules were detected by murine anti-myc antibody 9E10 (0.3µg/ml, Roche, Mannheim, Germany). M13 antibody (0.3µg/ml) (Amersham Pharmacia Biotech, Sweden) was used for the detection of phage particles. A horseradish peroxidase-conjugated antibody (anti-mouse IgG, 1:2000; Dako, Denmark) was used as secondary reagent. Tetramethylbenzidine was used as substrate (Sigma, Munich, Germany).

[000181] Antibody binding affinity by Surface Plasmon Resonance (SPR). SPR studies were performed using a BIAcore™ 3000 (BIAcore AB, St Albans, UK) as previously described (14, 35). HLA-A*0201-SLLMWITQC and HLA-A*0201-SLLMWITQV were enzymatically biotinylated by BirA enzyme on the C-terminal biotinylation site and immobilized to CM5 sensor chips via covalently coupled streptavidin. Kinetic constants were derived using the curve-fitting facility of the BIAevaluation program (Version 3.0, BIAcore) and rate equations derived from the simple 1:1 Langmuir binding model ($A+B \leftrightarrow AB$). Duplicates of each measurement were performed and averaged.

[000182] Flow cytometry. Stable transfected HLA-A0201 positive T2-cell lines expressing various HLA-A0201-restricted NY-ESO-1 peptides [so called mini-genes; 1a = NY-ESO-1 peptide 157-167, 1b = 157-165 and 1c = 155-163) or peptide pulsed (2h, 37°C) T2 cells (3×10^4) were used to verify binding-specificity of selected phages and Fabs, respectively (14). Cells were incubated with different Fabs at indicated concentrations in 100 µl phosphate-buffered saline-bovine serum albumin (PBS-BSA) (30 min, 4°C), washed in PBS-BSA and binding visualised by a two step procedure using antibody 9E10 [5µg/ml, (30min, 4°C)] and a PE-conjugated/anti-mouse IgG [dilution 1:10, Dianova, Hamburg, Germany; (30min, 4°C)]. Staining with w6/32 and BB7.2 antibody confirmed presence of HLA molecules.

[000183] An inhibition assay was set-up using a suboptimal concentration of PE conjugated 3M4E5-b tetramer. 3M4E5-Fab-tetramers were assembled by incubating 60µg biotinylated 3M4E5-Fab-monomers with 80µg of Streptavidin-conjugated R-PE [Invitrogen, Leiden, Netherland; (45min, 37°C or ON, 4°C)] at an optimal stoichiometric ratio of 1:4. The suboptimal 3M4E5 tetramer concentration was determined on T2-1b minigene cells or T2 cells pulsed with the respective NY-ESO-1 peptide (SLLMWITQV) (pulsing conditions: 2h 37°C, peptide concentration: 10^{-5} - 10^{-10} M). Cells were then incubated with 3M4E5 tetramer (1h, 4°C), washed twice with PBS-Tween 0.05% and varying

concentrations (0.0005-50 µg/ml) of candidate Fabs (45min, 4°C) added. All data were acquired by flow cytometry (FACScan, Becton Dickinson, Oxnard, CA) and analyzed with WinMDI program (J. Trotter, <http://facs.scripps.edu>).

[000184] T-cell inhibition. Assays were performed in a modified ELISPOT assay in triplicates on nitrocellulose-lined 96-well plates (MAHA S45 by Millipore, Bedford MA, USA). Wells were pre-coated overnight with an anti-IFN-γ capture antibody as recommended (Mabtech AB, Nacka, Sweden) and blocked (1h, 37°C) with RPMI containing 10% human serum. The CD8⁺ HLA-A2/ NY-ESO-1₁₅₇₋₁₆₅ specific T-cell clone was cultured as previously described (36). Target T2 cells were pulsed with 0.1 µg/ml of NY-ESO-1₁₅₇₋₁₆₅ peptide (1h, 37°C), stringently washed and incubated (1h, 37°C) with different concentrations (30, 3, 0.3, 0.003, 0.0003, 0.00003, 0 µg/ml) of HLA-A0201*/NY-ESO-1₁₅₇₋₁₆₅ complex specific or irrelevant Fab antibodies. CD8⁺-T-cells were co-cultured with target cells for 16h at 37°C (E:T of 1:1, ie. 8000:8000 cells per well). Plates were evaluated using an automated ELISPOT reader (Bioreader 3000, BioSys, Germany).

[000185] Generation and functional analysis of recombinant T-cell receptors. Candidate Fab antibodies were converted into scFv fragments, flanked by *NcoI* and *BamHI* restriction sites and cloned into the pBullet vector (37) containing human CD3 zeta and CD28 signalling domains (18). An anti-CEA scFv construct served as control. Retroviral transduction of CD3⁺ T cells with recombinant receptors was previously described in detail (18). Receptor expression was monitored by flow cytometry using PE labelled HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ tetramers. T-cells grafted with the recombinant immunoreceptors were co-cultivated in round bottom 96 well micro-titre plates (1.25–106104 grafted T cells/well) with HLA-A2/NY-ESO-1₁₅₇₋₁₆₅ positive and negative T2 cells (5000 cells/well). After 48 hours, culture supernatants were analysed for IFN-γ release using a sandwich ELISA [(coat mAb NIB42 (1µg/ml) (Pierce, Bonn Germany), detection by biotinylated mAb 4S.B3 (0.5µg/ml) (BD Bioscience, Heidelberg, Germany)]. The reaction was visualised by peroxidase-streptavidin (1:10,000) and ABTS (both Roche Diagnostics). Specific cytotoxicity of receptor grafted T cells against target cells was analysed using a colorimetric tetrazolium salt based assay indicating cell viability (EZ4U, Biomedica, Austria) as described (18).

[000186] References

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EXAMPLE 2

TUMOR CELL TARGETING

[000187] EL4 mouse lymphoma cell line is transfected with human HLA-A2 and with NY-ESO-1. These lymphoma cells are then capable of expressing the target peptide 157-165 in the context of human HL-A2. BL6 mice are challenged with the EL4-NY-ESO-1/HLA-A2 tumor cells. The 3M4E5 and T1 Fab antibodies are converted into fully human IgG1 antibodies and are used in these mice for tumor targeting using 111 Indium as tracer and in radioimmunotherapy using 177 Luthetium for cell killing. These studies are in line with studies of Klechevsky *et al* using immunotoxins with T-cell receptor-like specificity against human melanoma xenografts (Klechevsky E *et al.* (2008) *Cancer Res* 68(15):6360-6367). In those studies, T cell receptor-like Fab antibodies specific for melanoma-associated antigen MART-1(26-35) or gp100(280-288) presented by HLA-A201 were fused to truncated *Pseudomonas* exotoxin (PE38KDEL) and the immunotoxins used to target and kill HLA-A201 melanoma MART-1(+) and gp100(+) cell lines. The immunotoxins also significantly and discriminately inhibited human melanoma growth in severe-combined immunodeficient mice. These studies demonstrate that MHC class I/peptide complexes can serve as a specific target for passive immunotherapy of cancer.

[000188] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrated and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[000189] Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. An isolated specific binding member which recognizes an NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex on cells with an affinity greater than 100 times the affinity of TCR for said molecule.
2. The isolated specific binding member of claim 1 which recognizes an NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex.
3. The isolated specific binding member of claim 1 which is an antibody or antibody fragment comprising CDR domain sequences as set out in Figure 10, Figure 12 or Figure 13.
4. The isolated specific binding member of claim 3 which recognizes NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex on cells with an affinity greater than 1000 times the affinity of TCR for said molecule.
5. The isolated specific binding member of claim 1 which is an antibody or antibody fragment comprising a heavy chain and a light chain variable region comprising an amino acid sequence selected from the amino acid sequence set out in Figure 10, Figure 12 or Figure 13, or highly homologous variants thereof comprising 1 to 3 amino acid substitutions and retaining NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex binding with an affinity greater than 100 times the affinity of TCR.
6. An isolated antibody or fragment thereof which recognizes an NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex comprising a heavy chain and/or a light chain variable region comprising an amino acid sequence selected from the amino acid sequence set out in Figure 10, Figure 12 or Figure 13.
7. The isolated antibody of claim 5 which comprises a heavy chain having a variable region amino acid sequence set out in Figure 10, Figure 12 or Figure 13.
8. The isolated specific binding member of claim 1 which is an antibody or fragment thereof wherein said isolated antibody is the form of an antibody F(ab')₂, scFv fragment, diabody, triabody or tetrabody.
9. The isolated specific binding member of claim 1 which is an antibody, further comprising a detectable or functional label.

10. The isolated antibody of claim 9, wherein said detectable or functional label is a covalently attached drug.
11. The isolated antibody of claim 9, wherein said label is a radiolabel.
12. An isolated nucleic acid which comprises a sequence encoding an isolated specific binding member of claim 1.
13. A method of preparing a specific binding member or antibody as defined in any one of claims 1 to 8 which comprises expressing the nucleic acid of claim 12 under conditions to bring about expression of said binding member or antibody, and recovering the binding member or antibody.
14. A specific binding member or antibody according to any one of claims 1 to 11 for use in a method of treatment or diagnosis of the human or animal body.
15. A method of treatment of cancer in a human patient which comprises administering to said patient an effective amount of a specific binding member or antibody as defined in any one of claims 1 to 11.
16. A kit for the diagnosis of cancer in which NY-ESO-1 tumor antigen is expressed or presented, said kit comprising a specific binding member or antibody of any one of claims 1 to 11, optionally with reagents and/or instructions for use.
17. A kit for the diagnosis or monitoring of cancer selected from melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma cancer, said kit comprising a specific binding member or antibody of any one of claims 1 to 11, optionally with reagents and/or instructions for use.
18. A pharmaceutical composition comprising a specific binding member or antibody as defined in any one of claims 1 to 11, and optionally, a pharmaceutically acceptable vehicle, carrier or diluent.
19. A kit for the treatment of a tumor in a human patient, comprising a pharmaceutical dosage form of the pharmaceutical composition of claim 18, and a separate pharmaceutical dosage form comprising an additional anti-cancer agent selected from the group consisting of chemotherapeutic agents, radioimmunotherapeutic agents, and combinations thereof.

20. A unicellular host transformed with a recombinant DNA molecule comprising a DNA sequence or degenerate variant thereof, which encodes the specific binding member or antibody of any one of claims 1-8, or a fragment thereof, selected from the group consisting of:

- (A) the DNA sequence of FIGURE 11;
- (B) the DNA sequence of the heavy chain variable region sequence of FIGURE 11;
- (C) the DNA sequence comprising the CDR region sequences of the heavy chain and light chain of FIGURE 11;
- (D) DNA sequences that hybridize to any of the foregoing DNA sequences under standard hybridization conditions; and
- (E) DNA sequences that code on expression for an amino acid sequence encoded by any of the foregoing DNA sequences;

wherein said DNA sequence is operatively linked to an expression control sequence..

21. The unicellular host of Claim 20, wherein the unicellular host is selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, yeasts, CHO, YB/20, NSO, SP2/0, R1.1, B-W, L-M, COS 1, COS 7, BSC1, BSC40, and BMT10 cells, plant cells, insect cells, and human cells in tissue culture.

22. A method for detecting the presence of cancer in a mammal wherein said cancer is measured by determining the presence and/or amount of NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex:

A. contacting a biological sample from a mammal in which the presence of cancer is suspected with the binding member or antibody of any of claims 1 to 11 under conditions that allow binding of said NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex to said antibody to occur; and

B. detecting whether binding has occurred between said NY-ESO-1₁₅₇₋₁₆₅/MHC molecule or complex from said sample and the antibody;

wherein the detection of binding indicates that presence of cancer in said sample.

23. A method of preventing and/or treating cancer in mammals, comprising administering to a mammal a therapeutically effective amount of the pharmaceutical composition of claim 18 or the kit of claim 19.

24. A method for the treatment of cancer selected from melanoma, lung, esophageal, liver, gastric, prostate, ovarian, bladder and synovial sarcoma cancer in mammals, comprising administering to a

mammal a therapeutically effective amount of the pharmaceutical composition of claim 18 or the kit of claim 19.

25. The method of claim 23 or 24, wherein said pharmaceutical composition or said kit is administered systemically.

26. Use of a specific binding member or antibody of any one of claims 1 to 11 for the treatment or prevention of cancer in a mammal.

27. Use of a pharmaceutical composition according to claim 18 or the kit of claim 19, for the treatment or prevention of cancer in a mammal.

28. Use of a specific binding member or antibody of any one of claims 1 to 28 and 49-54 for the preparation of a medicament for the treatment or prevention of cancer in a mammal.

29. A method for detecting cancer in mammals comprising conducting diagnostic imaging, employing the specific binding member or antibody of claims 9 or 11.

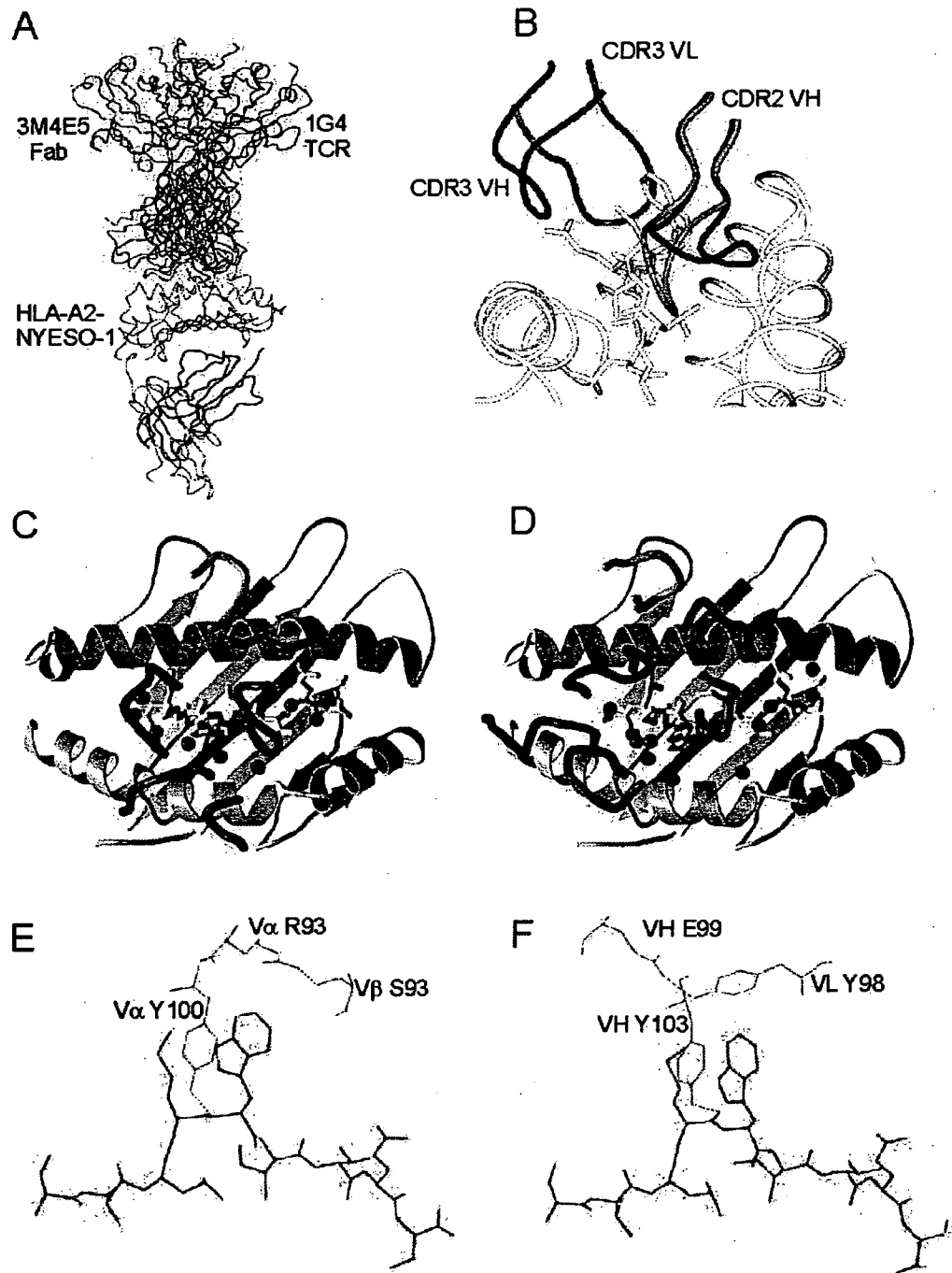
FIGURE 1

FIGURE 2

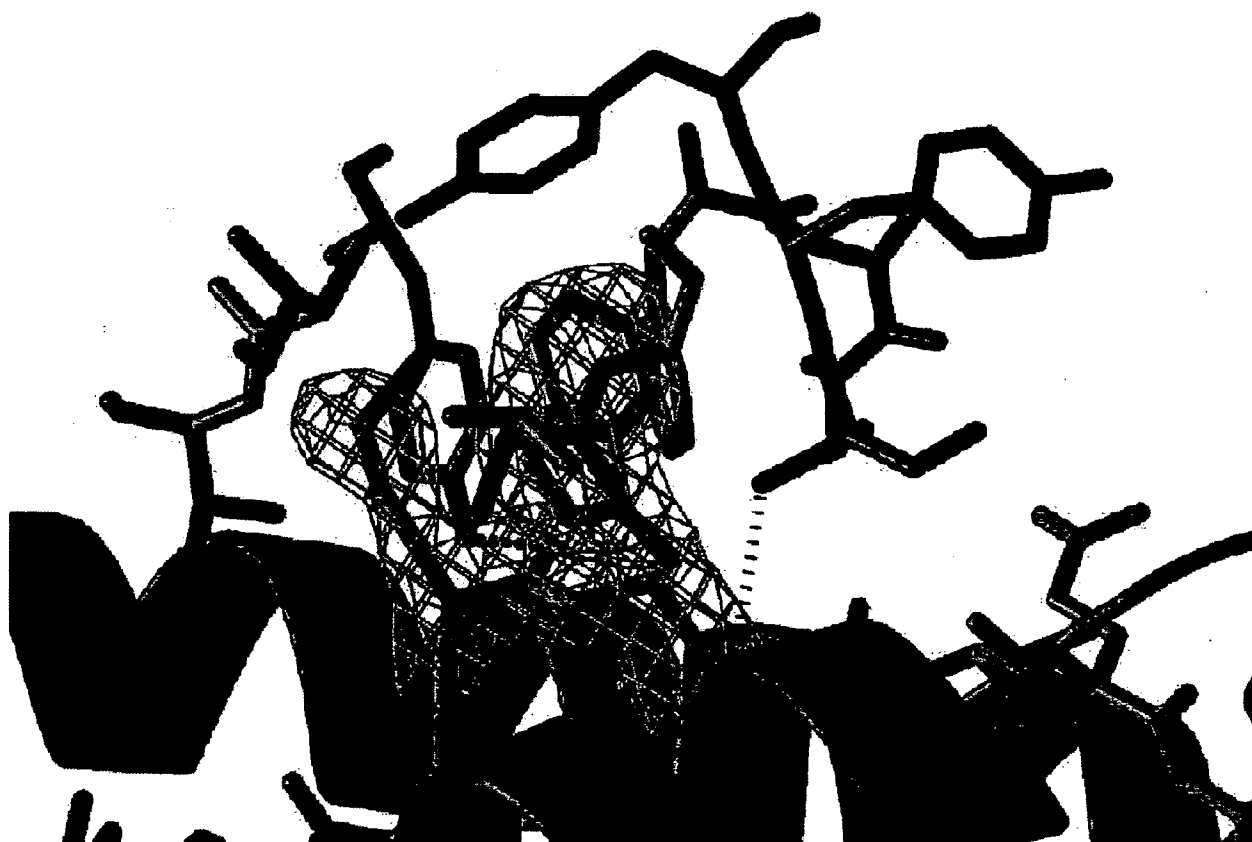


FIGURE 3

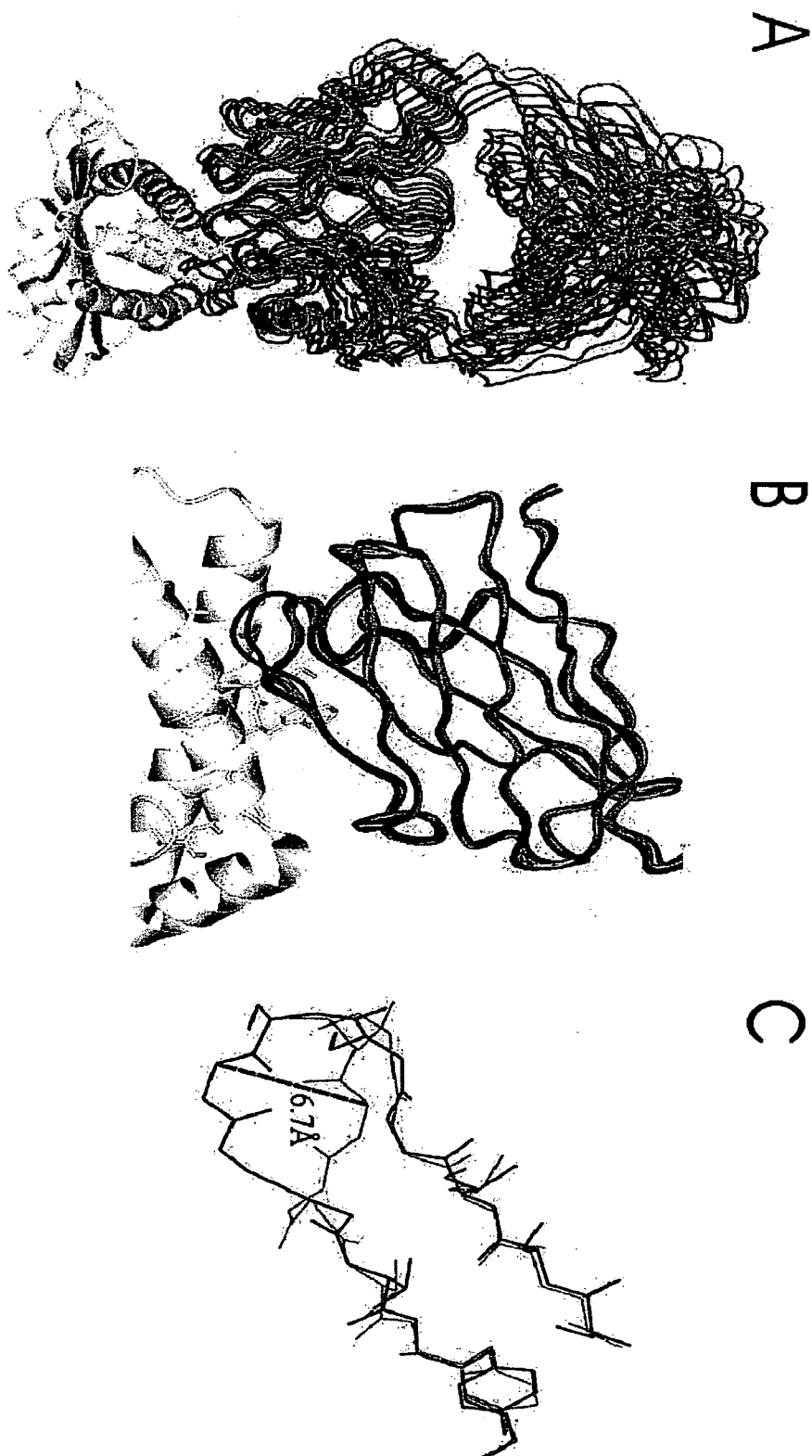


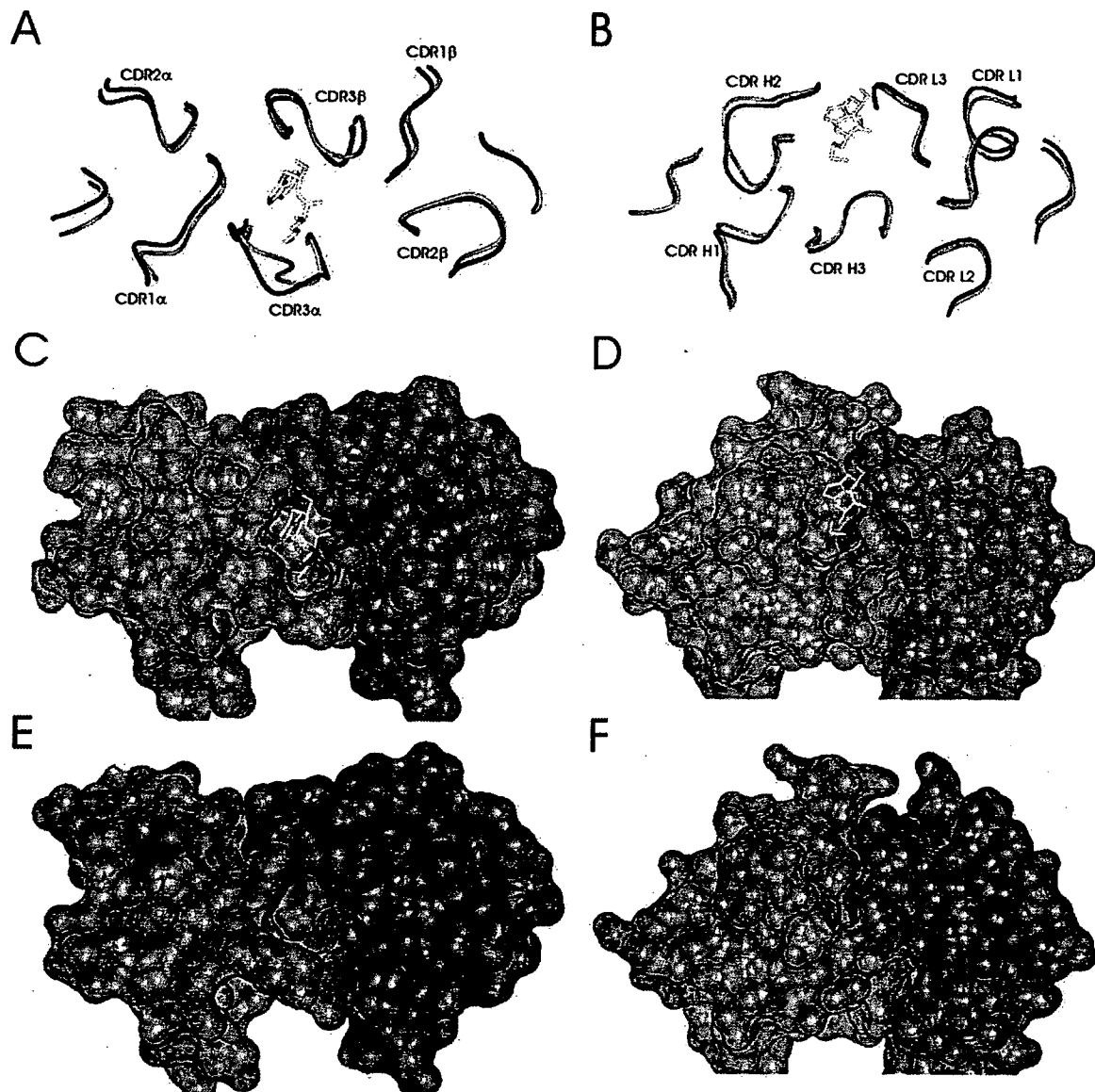
FIGURE 4

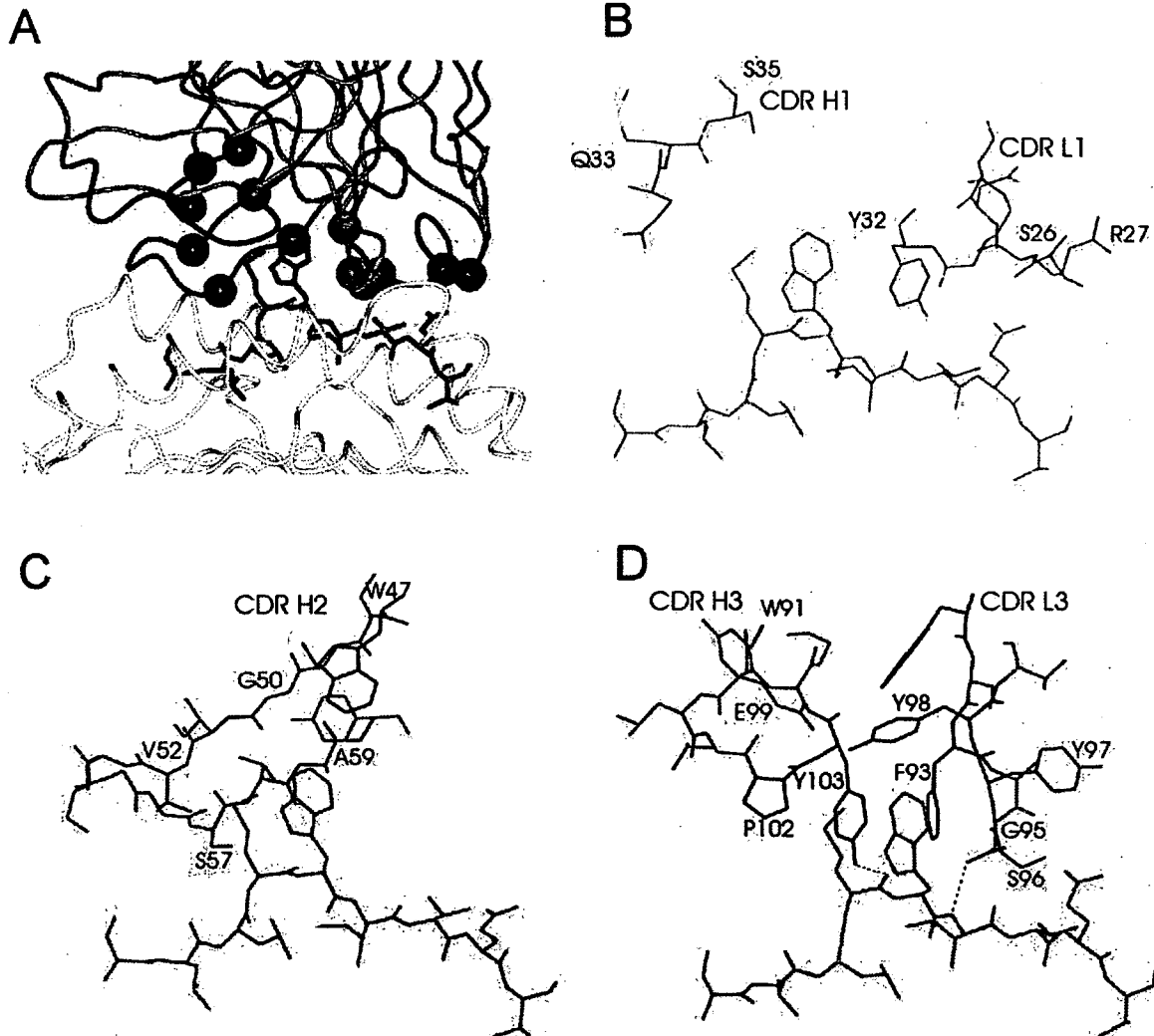
FIGURE 5

FIGURE 6

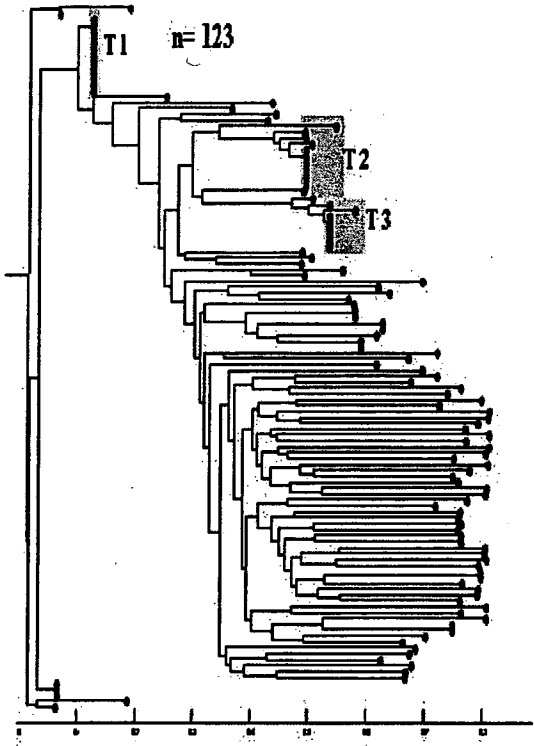


FIGURE 7

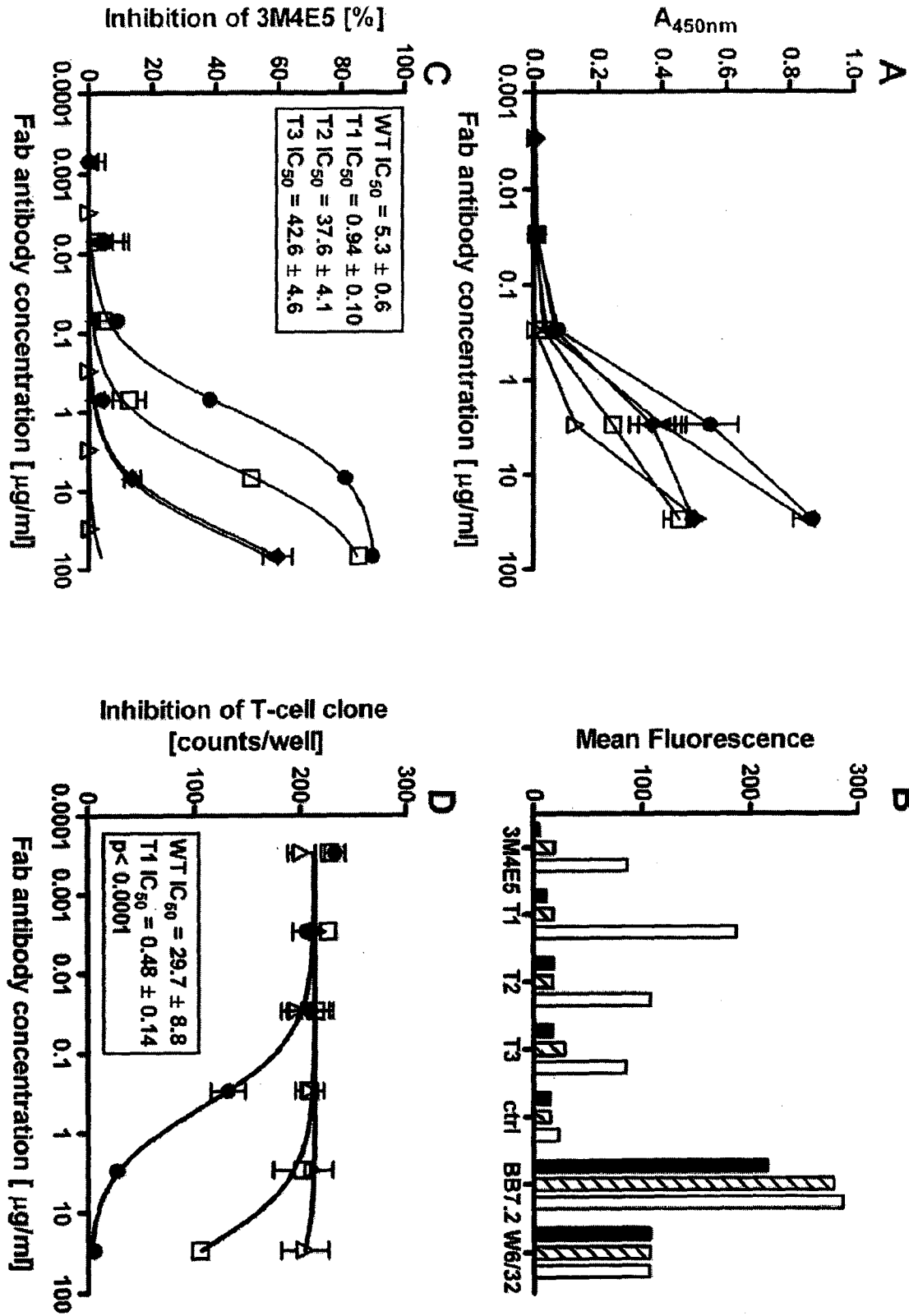


FIGURE 8

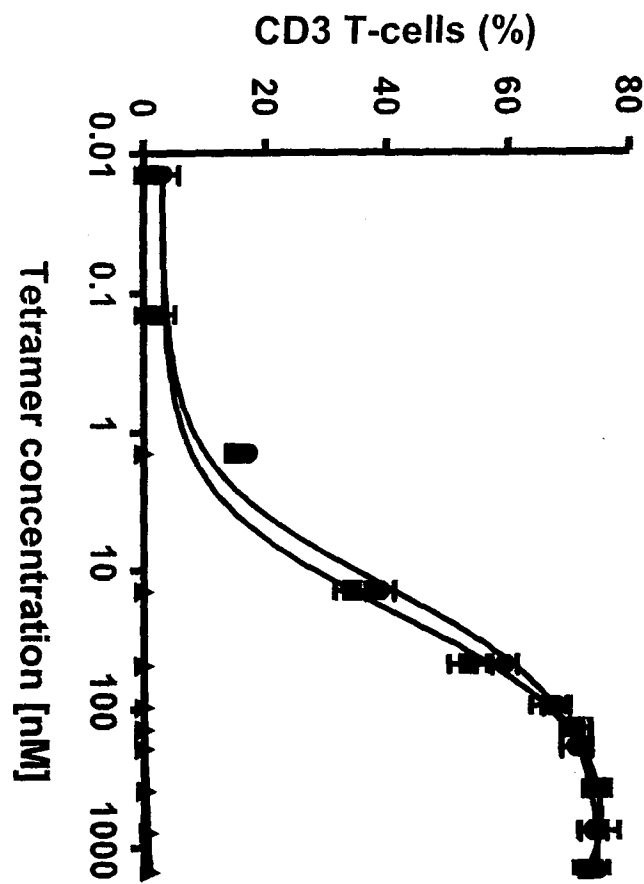


FIGURE 9

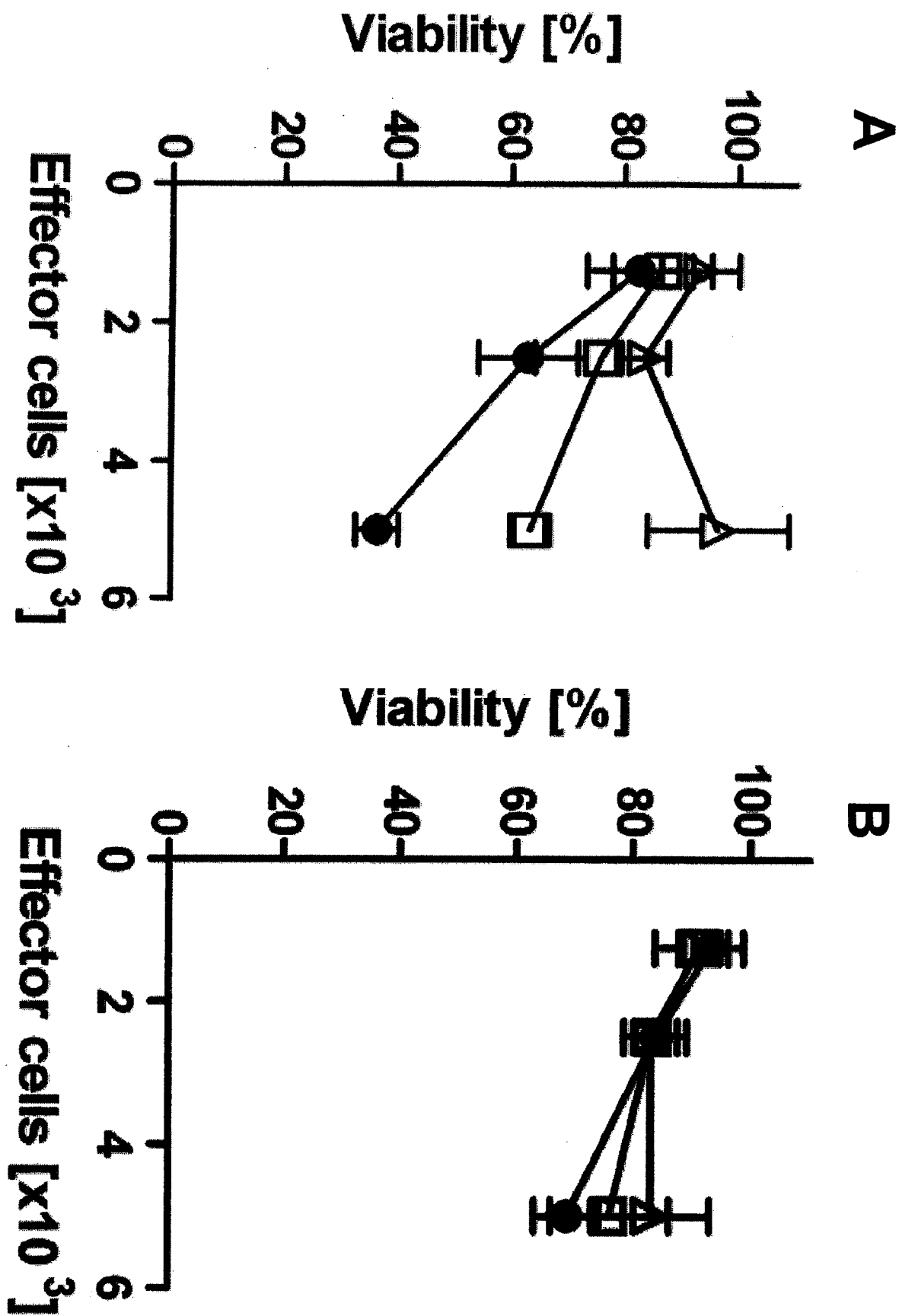


FIGURE 10**Amino-Acid sequence****Heavy chain**

WT 1 EVQLLESGGGLVQPGGSLRLSCAASG**FTTF**STYQMSWVRQAPGKGLEWVSGIVSSGGSTAY 60

|||||

T1 1 EVQLLESGGGLVQPGGSLRLSCAASG**FTTF**STYQMSWVRQAPGKGLEWVSGIVSSGGSTAY 60

WT 61 ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAGELLPYYG**MD**VWGQGT TTVTVSS 119

|||||

T1 61 ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAGELLPYYG**MD**VWGQGT TTVTVSS 119

Light chain

WT 1 QSELTQPRSVSGSPGQSVTISCTGT**SRDV**GGYNYVSWYQQHPGKAPKLIH**DV**IERSSGV 60

|||||

T1 1 QSELTQPRSVSGSPGQSVTISCTGT**ERDV**GGYNYVSWYQQHPGKAPKLIH**DV**IERSSGV 60

WT 61 PDRFSGSKSGNTASLTISGLQAEDEADYYC**WSFAG**SYVFGTGTDTVTL 109

|||||

T1 61 PDRFSGSKSGNTASLTISGLQAEDEADYYC**WSFAG**GYVFGTGTDTVTL 109

FIGURE 11**cDNA sequence****Heavy chain**

WT	1	GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTCAGCCTGGTGGTTCTTTACGTCTT	60
T1	1	GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTCAGCCTGGTGGTTCTTTACGTCTT	60
WT	61	TCTTGCGCTGCTTCGGATTCACTTTCTCTACTTACCAGATGTCTTGGGTTCGCCAAGCT	120
T1	61	TCTTGCGCTGCTTCGGATTCACTTTCTCTACTTACCAGATGTCTTGGGTTCGCCAAGCT	120
WT	121	CCTGGTAAAGGTTTGGAGTGGGTTTCTGGTATCGTTTCTTCTGGTGGCTCTACTGCTTAT	180
T1	121	CCTGGTAAAGGTTTGGAGTGGGTTTCTGGTATCGTTTCTTCTGGTGGCTCTACTGCTTAT	180
WT	181	GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC	240
T1	181	GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC	240
WT	241	TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGGGGAGCTA	300
T1	241	TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGGGGAGCTA	300
WT	301	CTTCCCTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCAAGC	357
T1	301	CTTCCCTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCAAGC	357

Light chain

WT	1	CAGAGCGAATTGACTCAGCCTCGCTCAGTGTCCGGGTCTCCTGGACAGTCAGTCACCATC	60
T1	1	CAGAGCGAATTGACTCAGCCTCGCTCAGTGTCCGGGTCTCCTGGACAGTCAGTCACCATC	60
WT	61	TCCTGCACTGGGACCAGCCGTGATGTTGGTGGTTATAACTATGTCTCCTGGTACCAACA	120
T1	61	TCCTGCACTGGGACCAGCCGTGATGTTGGTGGTTATAACTATGTCTCCTGGTACCAACA	120
WT	121	ACACCCAGGCAAAGCCCCAACTCATAATTGATGTCATAGAGCGGTCGTCAGGGGT	180
T1	121	ACACCCAGGCAAAGCCCCAACTCATAATTGATGTCATAGAGCGGTCGTCAGGGGT	180
WT	181	CCCTGATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATCTCTGGGCT	240
T1	181	CCCTGATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATCTCTGGGCT	240
WT	241	CCAGGCTGAGGATGAGGCTGATTATTATTGCTGGTCATTTCAGGCTCCTATTATGTCTT	300
T1	241	CCAGGCTGAGGATGAGGCTGATTATTATTGCTGGTCATTTCAGGCTCCTATTATGTCTT	300
WT	301	CGGGACAGGGACCGACGTCACCGTCCTC	328
T1	301	CGGGACAGGGACCGACGTCACCGTCCTC	328

T2 61 PDRFSGSKSGNTASLTISGLQAEDEADYYC**WSFAGGYV**FGTGTDTVTL 109

FIGURE 13**Amino-Acid sequence****Heavy chain**

WT 1 EVQLLESGGGLVQPGGSLRLSCAAS**GFTTFSTYQ**MSWVRQAPGKGLEWVSGIVSSGGSTAY 60

|||||
|||||

T3 1 EVQLLESGGGLVQPGGSLRLSCAAS**GFTTFSTYYM**DWVRQAPGKGLEWV**STIASSG**GETAY 60

WT 61 ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYC**AGELL**PYY**GMD**VWGQGTTVTVSS 119

|||||
|||||

T3 61 ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYC**AGSLL**PYY**GMD**VWGQGTTVTVSS 119

Light chain

WT 1 QSELTQPRSVSGSPGQSVTISCTGTS**RDVGG**YNYVSWYQQHPGKAPKLI**HD**VIERSSGV 60

|||||
|||||

T3 1 QSELTQPRSVSGSPGQSVTISCTGTE**RDVGG**YNYVSWYQQHPGKAPKLI**HD**VIERSSGV 60

WT 61 PDRFSGSKSGNTASLTISGLQAEDEADYYC**WSFAGS**YYVFGTGTDTVTL 109

|||||
|||||

T3 61 PDRFSGSKSGNTASLTISGLQAEDEADYYC**WSFAGG**YYVFGTGTDTVTL 109