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DESCRIPTION

Background

Immunotherapy of cancer

[0001] Cancer is a leading cause of premature deaths in the developed world. Immunotherapy of cancer aims to mount an effective immune response against tumour cells. This may be achieved by, for example, breaking tolerance against tumour antigen, augmenting anti-tumor immune responses, and stimulating local cytokine responses at the tumor site. The key effector cell of a long lasting anti-tumor immune response is the activated tumor specific effector T cell. Potent expansion of activated tumour-specific effector T cells can redirect the immune response towards the tumor. In this context, various immunosuppressive mechanisms induced by the tumor microenvironment suppress the activity of effector T cells. Several immunosuppressive mediators are expressed by the tumor cells. Such mediators inhibit T cell activation, either directly, or indirectly by inducing e.g. regulatory T cells (Treg) or myeloid-derived suppressor cells. Depleting, inhibiting, reverting or inactivating such regulatory cells may therefore provide anti-tumor effects and revert the immune suppression in the tumor microenvironment. Further, incomplete activation of effector T cells by, for example, dendritic cells can result in sub-optimally activated or anergic T cells, resulting in an inefficient anti-tumor response. In contrast, adequate induction by dendritic cells can generate a potent expansion of activated effector T cells, redirecting the immune response towards the tumor. In addition, Natural killer (NK) cells play an important role in tumor immunology by attacking tumor cells with down-regulated human leukocyte antigen (HLA) expression and by inducing antibody dependent cellular cytotoxicity (ADCC). Stimulation of NK cells may thus also reduce tumor growth.

Tumour-associated antigens

[0002] Tumor-associated antigens (TAA) are cell surface proteins selectively expressed on tumor cells. The term tumor-*associated* indicates that TAA are not completely tumor-specific, but are rather over-expressed on the tumor. A vast number of TAA have been described and used in various therapeutic rationales, including monoclonal antibodies, T cell redirecting therapies with TAA-CD3 bispecific antibodies, immunocytokines and antibody drug conjugates. Some well-studied TAA include the EGFR family molecules (HER2, HER3 and EGFR/HER1), VEGFR, EpCAM, CEA, PSA, PSMA, EphA2, gp100, GD2, MUC1, CD20, CD19, CD22 and CD33, summarized in (Cheever *et al.,* 2009).

[0003] 5T4 (also designated trophoblast glycoprotein, TPBG, M6P1 and Waif1) is a well-defined TAA originally identified by Professor Peter Stern, University of Manchester (Hole and Stern, 1988). It is an oncofetal antigen expressed in a high proportion of patients in a variety of malignancies, including non-small cell lung, renal, pancreas, prostate, breast, colorectal, gastric, ovarian and cervix cancers as well as in acute lymphocytic leukemia , and has also been shown to be expressed in tumor-initiating cells (Castro *et al.,* 2012; Damelin *et al.,* 2011; Elkord *et al.,* 2009; Southall *etal.,*

1990).

[0004] 5T4 expression is tumor-selective, with no or low expression in most normal tissues. In non-malignant tissue, 5T4 is mainly expressed in the placenta (trophoblast and amniotic epithelium) and at low levels in some specialised epithelia (Hole and Stern, 1988), as well as low at levels in other normal tissues (see US 2010/0021483). However, although low levels have been detected in some healthy tissue, the safety risk associated with this is considered low since expression levels in the tumor are considerably higher. This is supported by the fact that the phase III clinical programs, ANYARA and TroVax targeting 5T4 did not report severe 5T4-related toxicities.

[0005] Data from Stern et al. demonstrate that 5T4 regulates the functional activity of CXCR4 (Castro *et al.,* 2012; Southgate *et al.,* 2010). 5T4 binding antibodies or 5T4 knock-down resulted in inhibition of CXCR4-mediated cellular migration. The CXCR4 pathway is involved in tumor growth and metastasis. Therefore, targeting 5T4 in a CXCR4 inhibitory manner is likely to reduce tumor growth and/or spread.

CD137

[0006] CD137 (4-1BB, TNFRSF9) is a tumor necrosis factor (TNF) receptor (TNFR) superfamily member. Its role in cancer immunotherapy has been reviewed in e.g. (Bartkowiak and Curran, 2015). Activation of CD137 is dependent on receptor oligomerization (Rabu et al., 2005; Wyzgol et al., 2009) which is induced by binding to CD137L expressed as a trimer on the cell surface of antigen presenting cells (APCs) and other cell types. CD137 is expressed on various cell populations including activated $CD4^+$ and $CD8^+$ T cells, regulatory T cells (Treg), dendritic cells (DC), monocytes, mast cells, eosinophils and tumor endothelial cells. CD137 activation plays an important role in $CD8^+$ T cell activation and survival (Lee *et al.,* 2002; Pulle *et al.,* 2006). It sustains and augments effector functions and preferentially supports Th1 cytokine production (Shuford *et al.,* 1997). In $CD4^+$ T cells, CD137 stimulation initially results in activation and later in activation-induced cell death, which is thought to explain why CD137 agonistic antibodies have shown therapeutic effect in tumor immunity as well as in autoimmunity (Zhang, JCI, 2007, Sun, Trends Mol Med, 2003). CD137 has also been reported to suppress Treg function or convert Tregs to cytotoxic $CD4^+$ T-cells (Akhmetzyanova *et al.,* 2016; So *et al.,* 2008).

[0007] CD137 is upregulated on NK cells activated by cytokines or CD16 (FcγRIII) stimulation (ref in Melero, CCR 19 (5)1044-53, 2013). Activation of CD137 has been shown to increase antibody-dependent cellular cytotoxicity (ADCC) activity of NK cells in both murine and human cells (Kohrt 2012 and 2014 J Clin Invest, reviewed by Hout 2012, Oncoimm). Further, CD137 is expressed on APCs, such as DCs and macrophages, and stimulation of CD137 on these cell types may induce immune activation that can result in tumor immunity.

[0008] Agonistic CD137 antibody has been shown to activate endothelial cells in the tumor environment, leading to upregulation of ICAM-1 and VCAM-1 and improved T cell recruitment (Palazon, Cancer Res, 2011).

[0009] Several studies have demonstrated induction of tumor immunity by treatment with agonistic CD137 mAb in pre-clinical models. The mode of action may include various cell types, with $CD8^+$ T cells being one of the main effector cells involved in CD137-induced tumor immunity (Dubrot *et al.,* 2010; Gauttier *et al*., 2014; Kim *et al.,* 2001; McMillin *et al.,* 2006; Melero *et al*., 1997; Miller *et al.,* 2002; Sallin *et al.,* 2014; Taraban *et al.,* 2002; Uno *et al.,* 2006; Vinay and Kwon, 2012; Wilcox *et al*., 2002). In addition, it synergizes with several immunomodulators, including CpG, TRAIL, CD40, OX-40, DR5, PD-1/PD-L1, CTLA-4, Tim-3, IL-2 and IL-12 (Curran *et al*., 2011; Gray *et al.,* 2008; Guo *et al*., 2013; Kwong *et al*., 2013; Lee *et al.,* 2004; Morales-Kastresana *et al.,* 2013; Pan *et al*., 2002; St Rose *et al*., 2013; Uno *et al*., 2006; Wei *et al*., 2013; Westwood *et al*., 2010; Westwood *et al.,* 2014a; Westwood *et al.,* 2014b). An important role of CD137 in the induction and maintenance of tumor immunity is further supported by the findings indicating that CD137+ tumor infiltrating T cells are tumor-specific and effectively protect from tumor growth (Ye *et al.,* 2014).

[0010] Two CD137 antibodies are in clinical development. Urelumab (BMS-66513) is a fully human IgG4 antibody developed by Bristol-Myers Squibb. Several phase I and II studies in various indications are currently ongoing. A Phase II study with Urelumab as a second line therapy in metastatic melanoma was terminated in 2009 due to liver toxicity (Garber, 2011; Li and Liu, 2013). PF-05082566 is a fully human IgG2 antibody developed by Pfizer. It is currently in phase I development in lymphoma and various solid cancers and preliminary data suggest that it is well tolerated but with only modest anti-tumor effects.

[0011] Toxicity upon CD137 activation has been observed in patients as well as in mouse models (Ascierto *et al*., 2010; Dubrot *et al.,* 2010; Niu *et al*., 2007). The toxicity includes skin toxicities and liver toxicities manifested as increased aspartate amino transferase/alanine amino transferase ratio (ASAT/ALAT) levels and cytokine release. This suggests that either the toxicity requires CD137 mediated pre-activation of immune cell populations (likely T cells) or it depends on secondary effects caused by antidrug-antibodies (ADA) response, potentially forming aggregates of CD137 antibodies that may lead to enhanced cross-linking. The toxicities seen in mice are reversible and seem to depend on TNFa/CD8+ cells (Ascierto *et al.,* 2010). Toxicology studies in monkeys showed that both single and repeated dosing of up to 100mg/kg once weekly for four weeks was tolerable with no skin or liver toxicity detected (Ascierto 2010 Semin Onc).

[0012] TNFR family members are dependent on receptor cross-linking for activation to be induced. Such crosslinking may either be induced by the natural ligand expressed on the cell surface of cells or by recombinant, multimerized ligand. Alternatively, it may be induced by an antibody binding to the receptor and cross-linked by its Fc region bound to an Fcγ receptor (FcγR). This cross-linking dependence has been shown for various TNFR members, including DR5, GITR, CD27 and CD40 (Li and Ravetch, 2011; White *et al.,* 2011; Wilson *et al*., 2011a; Wilson *et al*., 2011b; Wyzgol *et al.,* 2009). An important role for the inhibitory FcγRIIB (CD32B) in activation by agonistic TNFR family antibodies was shown in some studies (Li and Ravetch, 2011; White *et al*., 2011; White *et al.,* 2013) whereas other data suggest that activation is induced by cross-linking of inhibitory as well as activating FcγRs (Li and Ravetch, 2011; Wilson *etal.,* 2011a).

[0013] Similar to other TNFR members, activation of CD137 is dependent on receptor oligomerization. Hexamers of CD137L effectively induce CD137 activation, whereas monomeric or trimeric CD137L does not (Rabu *et al.,* 2005; Wyzgol *et al.,* 2009). Thus, it is likely that CD137

agonistic antibodies require cross-linking, e.g. via FcγR for effective activation to occur *in vivo.* However, in contrast to other TNFR members, FcγRII is not critical for induction of tumor immunity by CD137, whereas FcγRIII impairs tumor immunity (Sallin *etal.,* 2014; Sanmamed *etal.,* 2015) in mouse studies.

[0014] The translational relevance of the role of various FcγR in activation of CD137 and other TNFR superfamily members is uncertain, since the human FcγR distribution as well as the affinity of different IgG isotypes to different FcγR differ between mice and humans.

[0015] US 2015/307620 is entitled "Linked immunotherapeutic agonists that costimulate multiple pathways". WO 2015/156268 (also published as EP-A-3130606) is entitled "Immunoactivating antigen-binding molecule" and relates to a bispecific antibody comprising a cancer-specific antigen-binding domain and a human CD137-binding domain. WO 2014/116846 is entitled "Methods and compositions for modulating an immune response". WO 01/56603 is entitled "CD40-binding APC-activating molecules". Makkouk et al., 2016 is entitled "Rationale for anti-CD137 cancer immunotherapy". Arndt et al., 2013 is entitled "Costimulation improves the killing capability of T cells redirected to tumor cells expressing low levels of CD33: description of a novel modular targeting system". Hinner et al., 2015 is a poster entitled "Costimulatory T cell engagement via a novel bispecific anti-CD137 /anti-HER2 protein". Hinner et al., 2015 is an abstract entitled "Abstract B023: Costimulatory T-cell engagement via a novel bispecific anti-CD137 /anti-HER2 protein based on Anticalin® technology". WO 2016/110584 is entitled "Agonistic TNF receptor binding agents". WO 2016/115/274 is entitled "Multispecific immunomodulatory antigen-binding constructs". WO 2016/185016 is entitled "Novel polypeptides". Vezys et al., 2011 is entitled "4-1BB Signaling Synergizes with Programmed Death Ligand 1 Blockade To Augment CD8 T Cell Responses during Chronic Viral Infection". Imura et al., 1997 is entitled "OX40 expressed on fresh leukemic cells from adult T-cell leukemia patients mediates cell adhesion to vascular endothelial cells: implication for the possible involvement of OX40 in leukemic cell infiltration". Yamauchi et al., 2005 is entitled "The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma". US 2007/161080 is entitled "Antibodies". WO 2013/041687 is entitled "Bispecific binding molecules for 5T4 and CD3". Forsberg et al., 2010 is entitled "Naptumomab Estafenatox, an Engineered Antibody-superantigen Fusion Protein With Low Toxicity and Reduced Antigenicity". White et al., 2015 is entitled "Conformation of the Human Immunoglobulin G2 Hinge Imparts Superagonistic Properties to Immunostimulatory Anticancer Antibodies". Moraga et al., 2015 is entitled "Tuning Cytokine Receptor Signaling by Re-orienting Dimer Geometry with Surrogate Ligands". Cheng et al., 2004 is entitled "Individualized Patient Dosing in Phase I Clinical Trials: The Role of Escalation With Overdose Control in PNU-214936".

[0016] Despite progress in the development of immunotherapies for the treatment of various cancers over the last decade, there remains a need for new and efficacious agents.

[0017] Accordingly, the present invention seeks to provide improved polypeptide-based therapies for the treatment of cancer.

Summary of the Invention

[0018] A first aspect of the invention provides a bispecific antibody comprising a first binding domain, designated B1, which is capable of binding specifically to CD137, and a second binding domain, designated B2, which is capable of specifically binding to a tumour cell-associated antigen, wherein the tumour cell-associated antigen is 5T4;

wherein binding domain B1 comprises CDRL1 corresponding to SEQ ID NO: 54, CDRL2 corresponding to SEQ ID NO: 55, and CDRL3 corresponding to SEQ ID NO: 83, and

wherein binding domain B1 comprises CDRH1 corresponding to SEQ ID NO: 62 or a variant of CDRH1 having only one amino acid mutation relative to SEQ ID NO: 62, CDRH2 corresponding to SEQ ID NO: 69, and CDRH3 corresponding to SEQ ID NO: 76; and

wherein binding domain B2 comprises CDRL1 corresponding to SEQ ID NO: 54 or a variant of CDRL1 having only one amino acid mutation relative to SEQ ID NO: 54, CDRL2 corresponding to SEQ ID NO: 55, and CDRL3 corresponding to SEQ ID NO: 58, and

wherein binding domain B2 comprises CDRH1 corresponding to SEQ ID NO: 46, CDRH2 corresponding to SEQ ID NO: 48, and CDRH3 corresponding to SEQ ID NO: 52.

[0019] Such bispecific compounds comprising one immune-activating moiety, that is a CD137 agonist, and one tumor-targeting moiety, that is a 5T4 binder, can be used to establish a highly effective and safe cancer immunotherapy.

[0020] Various types of tumor-localizing immunotherapeutic molecules, such as immunocytokines and bispecific antibodies have shown beneficial immune activation and inhibition of tumor growth in preclinical studies as well as in the clinic (reviewed in Kiefer and Neri, 2016).

[0021] To avoid systemic toxicity by CD137 activating agents, yet obtain high efficacy in the tumor area, the designs of the molecular format of a CD137 agonist may be optimised. For example, a good efficacy/safety profile can be obtained by a TAA-CD137 bispecific antibody that requires crosslinking by binding to the TAA for CD137 activation to occur. Then, preactivated, CD137-expressing T cells residing in the tumor will preferentially be activated, whereas CD137 expressing cells in other tissues will not. This would allow focused activation of the relevant, tumor-specific T cells while limiting toxicity induced by generalised CD137 activation ('activation' in this context being a net immune activation that results in a tumor-directed T cell response, for example by down-regulation of Tregs suppressive function and/or upregulation of effector T cell function).

[0022] The clinical progress with immunocytokines has so far not been impressive and the side effects still remain since the tumor-binding entity only confers limited tumor localization, with the bulk of the immunocytokine ending up in other compartments. Bispecific antibodies that restrict the activity to the tumor as described in this invention would provide a clear advantage over immunocytokines since they are inactive in the absence of tumors.

[0023] Further, the bispecific polypeptides of the invention provide a distinct advantage over bispecific antibodies targeting CD3. CD3-targeting bispecific molecules use T cells as effector cells and are capable of activating T cells independent of TAA binding. Thus, they do not activate tumor

specific T-cells in particular. The resulting anti-tumor effects are therefore not likely to generate a long lasting anti-tumor immunity. In addition, since CD3 is expressed on all T cells, systemic T cell activation is associated with toxicity issues. In contrast, the bispecific antibodies of the invention have the potential to selectively activate tumor specific T-cells and generate a long lasting tumour immunity.

Structure of bispecific polypeptide

[0024] A "polypeptide" is used herein in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or other peptidomimetics. The term "polypeptide" thus includes short peptide sequences as well as longer polypeptides and proteins. As used herein, the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including both D or L optical isomers, and amino acid analogs and peptidomimetics.

[0025] The term "bispecific" as used herein means the polypeptide is capable of specifically binding at least two target entities.

[0026] The polypeptide is a bispecific antibody (numerous examples of which are described in detail below).

[0027] Thus, the first and/or second binding domains may be selected from the group consisting of antibodies and antigen-binding fragments thereof.

[0028] By "an antibody or an antigen-binding fragment thereof" we include substantially intact antibody molecules, as well as chimaeric antibodies, humanised antibodies, isolated human antibodies, single chain antibodies, bispecific antibodies, antibody heavy chains, antibody light chains, homodimers and heterodimers of antibody heavy and/or light chains, and antigen-binding fragments and derivatives of the same. Suitable antigen-binding fragments and derivatives include Fv fragments (*e.g.* single chain Fv and disulphide-bonded Fv) and Fab-like fragments (*e.g.* Fab fragments, Fab' fragments and F(ab)2 fragments). The potential advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Moreover, antigen-binding fragments such as Fab, Fv, ScFv and dAb antibody fragments can be expressed in and secreted from *E. coli,* thus allowing the facile production of large amounts of the said fragments.

[0029] In one embodiment, the antigen-binding fragment is selected from the group consisting of: Fv fragments (such as a single chain Fv fragment, or a disulphide-bonded Fv fragment), and Fab-like fragments (such as a Fab fragment; a Fab' fragment or a $F(ab)_2$ fragment).

[0030] For example, the first binding domain (B1) and/or the second binding domain (B2) may comprise or consist of a Fab fragment.

[0031] Alternatively, or in addition, the first binding domain (B1) and/or the second binding domain (B2) may comprise or consist of an Fv fragment (such as an scFv or di-sulphide bridged Fv). Where the binding domain is an scFv, the VH and VL regions therein may be joined by a linker sequence,

for example:
GGGGSGGGGSGGGGS [SEQ ID NO: 93]

[0032] It will be appreciated by persons skilled in the art that such scFv polypeptides may be glycosylated, for example *N*-glycosylated, on one or more amino acid residues.

[0033] The phrase "an antibody or an antigen-binding fragment thereof" is also intended to encompass antibody mimics (for example, non-antibody scaffold structures that have a high degree of stability yet allow variability to be introduced at certain positions). Those skilled in the art of biochemistry will be familiar with many such molecules, as discussed in Gebauer & Skerra, 2009, Curr Opin Chem Biol 13(3): 245-255. Exemplary antibody mimics include: affibodies (also called Trinectins; Nygren, 2008, FEBS J, 275, 2668-2676); CTLDs (also called Tetranectins; Innovations Pharmac. Technol. (2006), 27-30); adnectins (also called monobodies; Meth. Mol. Biol., 352 (2007), 95-109); anticalins (Drug Discovery Today (2005), 10, 23-33); DARPins (ankyrins; Nat. Biotechnol. (2004), 22, 575-582); avimers (Nat. Biotechnol. (2005), 23, 1556-1561); microbodies (FEBS J, (2007), 274, 86-95); peptide aptamers (Expert. Opin. Biol. Ther. (2005), 5, 783-797); Kunitz domains (J. Pharmacol. Exp. Ther. (2006) 318, 803-809); affilins (Trends. Biotechnol. (2005), 23, 514-522); affimers (Avacta Life Sciences, Wetherby, UK).

[0034] Also described herein are chimaeric T-cell receptors (also known as chimaeric T cell receptors, chimaeric immunoreceptors, and chimaeric antigen receptors or CARs) (see Pule et al., 2003, Cytotherapy 5(3):211-26. These are engineered receptors, which graft an arbitrary specificity onto an immune effector cell. Typically, CARs are used to graft the specificity of a monoclonal antibody onto a T cell; with transfer of their coding sequence facilitated by retroviral vectors. The most common form of such molecules is fusions comprising a single-chain variable fragment (scFv) derived from a monoclonal antibody fused to CD3-zeta transmembrane and endodomain. When T cells express this fusion molecule, they recognize and kill target cells that express the transferred monoclonal antibody specificity.

[0035] Persons skilled in the art will further appreciate that the invention also encompasses modified versions of antibodies, whether existing now or in the future, e.g. modified by the covalent attachment of polyethylene glycol or another suitable polymer (see below).

[0036] Methods of generating antibodies and antibody fragments are well known in the art. For example, antibodies may be generated via any one of several methods which employ induction of *in vivo* production of antibody molecules, screening of immunoglobulin libraries (Orlandi et al, 1989. Proc. Natl. Acad. Sci. U.S.A. 86:3833-3837; Winter et al., 1991, Nature 349:293-299) or generation of monoclonal antibody molecules by cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the Epstein-Barr virus (EBV)-hybridoma technique (Kohler et al., 1975. Nature 256:4950497; Kozbor et al., 1985. J. Immunol. Methods 81:31-42; Cote et al., 1983. Proc. Natl. Acad. Sci. USA 80:2026-2030; Cole etal., 1984. Mol. Cell. Biol. 62:109-120).

[0037] Suitable methods for the production of monoclonal antibodies are also disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", J G R Hurrell (CRC Press, 1982).

[0038] Likewise, antibody fragments can be obtained using methods well known in the art (see, for example, Harlow & Lane, 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory, New York). For example, antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (*e.g.* Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Alternatively, antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods.

[0039] It will be appreciated by persons skilled in the art that for human therapy or diagnostics, human or humanised antibodies are preferably used. Humanised forms of non-human (*e.g.* murine) antibodies are genetically engineered chimaeric antibodies or antibody fragments having preferably minimal-portions derived from non-human antibodies. Humanised antibodies include antibodies in which complementary determining regions of a human antibody (recipient antibody) are replaced by residues from a complementary determining region of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired functionality. In some instances, Fv framework residues of the human antibody are replaced by corresponding non-human residues. Humanised antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported complementarity determining region or framework sequences. In general, the humanised antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the complementarity determining regions correspond to those of a non-human antibody and all, or substantially all, of the framework regions correspond to those of a relevant human consensus sequence. Humanised antibodies optimally also include at least a portion of an antibody constant region, such as an Fc region, typically derived from a human antibody (see, for example, Jones et al., 1986. Nature 321:522-525**;** Riechmann et al., 1988, Nature 332:323-329; Presta, 1992, Curr. Op. Struct. Biol. 2:593-596).

[0040] Methods for humanising non-human antibodies are well known in the art. Generally, the humanised antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues, often referred to as imported residues, are typically taken from an imported variable domain. Humanisation can be essentially performed as described (see, for example, Jones et al., 1986, Nature 321:522-525; Reichmann et al., 1988. Nature 332:323-327; Verhoeyen et al., 1988, Science 239:1534-1536l; US 4,816,567) by substituting human complementarity determining regions with corresponding rodent complementarity determining regions. Accordingly, such humanised antibodies are chimaeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanised antibodies may be typically human antibodies in which some complementarity determining region residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies.

[0041] Human antibodies can also be identified using various techniques known in the art, including phage display libraries (see, for example, Hoogenboom & Winter, 1991, J. Mol. Biol. 227:381; Marks et al., 1991, J. Mol. Biol. 222:581; Cole et al., 1985, In: Monoclonal antibodies and Cancer Therapy, Alan R. Liss, pp. 77; Boerner et al., 1991. J. Immunol. 147:86-95).

[0042] It will be appreciated by persons skilled in the art that the bispecific antibodies of the present

invention may be of any suitable structural format.

[0043] Thus, in exemplary embodiments of the bispecific antibodies of the invention:

1. (a) binding domain B1 and/or binding domain B2 is an intact IgG antibody (or, together, form an intact IgG antibody);
2. (b) binding domain B1 and/or binding domain B2 is an Fv fragment (e.g. an scFv); and/or
3. (c) binding domain B1 and/or binding domain B2 is a Fab fragment.

[0044] It will be appreciated by persons skilled in the art that the bispecific antibody may comprise a human Fc region, or a variant of a said region, where the region is an IgG1, IgG2, IgG3 or IgG4 region, preferably an IgG1 or IgG4 region.

[0045] Engineering the Fc region of a therapeutic monoclonal antibody or Fc fusion protein allows the generation of molecules that are better suited to the pharmacology activity required of them (Strohl, 2009, Curr Opin Biotechnol 20(6):685-91).

(a) *Engineered Fc regions for increased half-life*

[0046] One approach to improve the efficacy of a therapeutic antibody is to increase its serum persistence, thereby allowing higher circulating levels, less frequent administration and reduced doses.

[0047] The half-life of an IgG depends on its pH-dependent binding to the neonatal receptor FcRn. FcRn, which is expressed on the surface of endothelial cells, binds the IgG in a pH-dependent manner and protects it from degradation.

[0048] Some antibodies that selectively bind the FcRn at pH 6.0, but not pH 7.4, exhibit a higher half-life in a variety of animal models.

[0049] Several mutations located at the interface between the CH2 and CH3 domains, such as T250Q/M428L (Hinton et al., 2004, J Biol Chem. 279(8):6213-6) and M252Y/S254T/T256E + H433K/N434F (Vaccaro et al., 2005, Nat. Biotechnol. 23(10):1283-8), have been shown to increase the binding affinity to FcRn and the half-life of IgG1 *in vivo.*

(b) *Engineered Fc regions for altered effector function*

[0050] To ensure lack of CD137 activation in the absence of the tumour antigen, the Fc portion of the bispecific antibody should bind with no or very low affinity to FcγR, since FcγR-mediated crosslinking of a CD137 antibody may induce activation. By "very low affinity" we include that the Fc portion exhibits at least 10 times reduced affinity to FcγRI, FcgRII and III compared to wild-type IgG1, as determined by the concentration where half maximal binding is achieved in flow cytometric

analysis of FcγR expressing cells (Hezareh et al., 2001, J Virol, 75(24):12161-8) or by FcγR ELISA (Shields et al., 2001, J Biol Chem. 276(9):6591-604).

[0051] Another factor to take into account is that engagement of FcγR's may also induce antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) of cells coated with antibodies. Thus, to ensure tumor-dependent CD137 activation as well as to avoid depletion of CD137 expressing, tumor-reactive T effector cells, the isotype of a TAA-CD137 bispecific antibody should preferably be silent.

[0052] The four human IgG isotypes bind the activating Fcγ receptors (FcγRI, FcγRIIa, FcγRIIIa), the inhibitory FcγRIIb receptor, and the first component of complement (C1q) with different affinities, yielding very different effector functions (Bruhns et al., 2009, Blood. 113(16):3716-25, the disclosures of which are incorporated herein by reference). IgG1 molecules have the highest affinity and capacity to induce effector functions, whereas IgG2, IgG3 and IgG4 are less effective (Bruhns, 2012; Hogarth and Pietersz, 2012; Stewart et al., 2014) (Wang 2015 Front Im; Vidarson 2014 Fron Imm). In addition, certain mutations in the Fc region of IgG1 dramatically reduces FcγR affinity and effector function while retaining neonatal FcR (FcRn) interaction (Ju and Jung, 2014; Leabman et al., 2013; Oganesyan et al., 2008; Sazinsky et al., 2008).

[0053] The most widely used IgG1 mutants are N297A alone or in combination with D265A, as well as mutations at positions L234 and L235, including the so-called "LALA" double mutant L234A/L235A. Another position described to further silence IgG1 by mutation is P329 (see US 2012/0251531).

[0054] Thus, choosing a mutated IgG1 format with low effector function but retained binding to FcRn may result in a bispecific antibody with 5T4-dependent activation of CD137, and exhibiting a favorable efficacy/safety profile and good PK properties.

[0055] Advantageously, the polypeptide is incapable of inducing antibody dependent cell cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and/or complement-dependent cytotoxicity (CDC). By "incapable" we include that the ability of the polypeptide to induce ADCC, *etc.,* is at least 10-fold lower than compared to wild-type IgG1 as shown by e.g. monocyte-dependent ADCC or CDC assays described by Hezareh *et al.* 2001 *(supra).*

[0056] In one embodiment, the Fc region may be a variant of a human IgG1 Fc region comprising a mutation at one or more of the following positions:
L234, L235, P239, D265, N297 and/or P329.

[0057] Advantageously, alanine may be present at the mutated positions(s).

[0058] Optionally, the IgG1 variant may be a variant of a human IgG1 Fc region comprising mutations L234A and L235A (*i.e.* the LALA double mutant; see SEQ ID NO: 86).

[0059] It will be appreciated by persons skilled in the art that the bispecific polypeptides of the invention may be of several different structural formats (for example, see Chan & Carter, 2016, Nature Reviews Immunology 10, 301-316).

[0060] In exemplary embodiments, the bispecific antibody is selected from the groups consisting of:

1. (a) bivalent bispecific antibodies, such as IgG-scFv bispecific antibodies (for example, wherein B1 is an intact IgG and B2 is an scFv attached to B1 at the N-terminus of a light chain and/or at the C-terminus of a light chain and/or at the N-terminus of a heavy chain and/or at the C-terminus of a heavy chain of the IgG, or *vice versa)*;
2. (b) monovalent bispecific antibodies, such as a *DuoBody*® (Genmab AS, Copenhagen, Denmark) or 'knob-in-hole' bispecific antibody (for example, an scFv-KIH, scFv-KIH^r, a BiTE-KIH or a BiTE- KIH^r (see Xu et al., 2015, mAbs 7(1):231-242);
3. (c) scFv$_2$-Fc bispecific antibodies (such as *ADAPTIR*™ bispecific antibodies from Aptevo Therapeutics Inc, Seattle, US);
4. (d) BiTE/scFv$_2$ bispecific antibodies;
5. (e) DVD-Ig bispecific antibodies or other IgG-FAb, FAb-IgG bispecific antibodies regardless of bivalency or linkers/ connectors employed;
6. (f) DART-based bispecific antibodies (for example, DART$_2$-Fc, DART$_2$-Fc or DART);
7. (g) DNL-Fab$_3$ bispecific antibodies; and
8. (h) scFv-HSA-scFv bispecific antibodies.

[0061] For example, the bispecific antibody may be an IgG-scFv antibody (see Figure 1). The IgG-scFv antibody (and, specifically, the scFv domain therein) may be in either VH-VL or VL-VH orientation. In one embodiment, the scFv may be stabilised by a S-S bridge between VH and VL.

[0062] In an alternative embodiment, the bispecific antibody may be an scFv$_2$-Fc antibody, for example a dimer wherein each polypeptide comprises, from the N-terminus to C-terminus, a first scFv, a hinge domain, an Fc domain and a second scFv (see Figure 1).

[0063] In one embodiment, binding domain B1 and binding domain B2 are fused directly to each other.

[0064] In an alternative embodiment, binding domain B1 and binding domain B2 are joined via a polypeptide linker. For example, a polypeptide linker may be a short linker peptide between about 10 to about 25 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa.

[0065] Thus, the linker may be selected from the group consisting of the amino acid sequence SGGGGSGGGGS (SEQ ID NO: 87), SGGGGSGGGGSAP (SEQ ID NO: 88), NFSQP (SEQ ID NO: 89), KRTVA (SEQ ID NO: 90), GGGSGGGG (SEQ ID NO: 91), GGGGSGGGGS, (SEQ ID NO: 92), GGGGSGGGGSGGGGS (SEQ ID NO: 93), THTCPPCPEPKSSDK (SEQ ID NO: 140), GGGS (SEQ ID NO: 141), EAAKEAAKGGGGS (SEQ ID NO: 142), EAAKEAAK (SEQ ID NO: 143), or (SG)m, where m = 1 to 7.

[0066] In a further embodiment, binding domain B1 and binding domain B2 are separated by

immunoglobulin constant regions (such as an Fc region) on a polypeptide.

[0067] The term "amino acid" as used herein includes the standard twenty genetically-encoded amino acids and their corresponding stereoisomers in the 'D' form (as compared to the natural 'L' form), omega-amino acids other naturally-occurring amino acids, unconventional amino acids (*e.g.* α,α-disubstituted amino acids, N-alkyl amino acids, *etc.*) and chemically derivatised amino acids (see below).

[0068] When an amino acid is being specifically enumerated, such as "alanine" or "Ala" or "A", the term refers to both $_{L}$-alanine and $_{D}$-alanine unless explicitly stated otherwise. Other unconventional amino acids may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded amino acid residue, where appropriate, is represented by a single letter designation, corresponding to the trivial name of the conventional amino acid.

[0069] In one embodiment, the antibody polypeptides as defined herein comprise or consist of $_{L}$-amino acids.

[0070] It will be appreciated by persons skilled in the art that the antibody polypeptides of the invention may comprise or consist of one or more amino acids which have been modified or derivatised.

[0071] Chemical derivatives of one or more amino acids may be achieved by reaction with a functional side group. Such derivatised molecules include, for example, those molecules in which free amino groups have been derivatised to form amine hydrochlorides, *p*-toluene sulphonyl groups, carboxybenzoxy groups, *t*-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatised to form salts, methyl and ethyl esters or other types of esters and hydrazides. Free hydroxyl groups may be derivatised to form O-acyl or O-alkyl derivatives. Also included as chemical derivatives are those peptides which contain naturally occurring amino acid derivatives of the twenty standard amino acids. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine and ornithine for lysine. Derivatives also include peptides containing one or more additions or deletions as long as the requisite activity is maintained. Other included modifications are amidation, amino terminal acylation (*e.g.* acetylation or thioglycolic acid amidation), terminal carboxylamidation (*e.g.* with ammonia or methylamine), and the like terminal modifications.

[0072] Alternatively, or in addition, one or more amino acid may be glycosylated, such as *N*-linked glycosylation (in which glycan moieties are attached to a nitrogen of asparagine or arginine side chains) and/or O-linked glycosylation (in which glycan moieties are attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline). Methods for the production of glycosylated antibodies are well known in the art (for example, see Jefferis, 2009, Nature Reviews Drug Discovery 8:226-234).

[0073] It will be further appreciated by persons skilled in the art that peptidomimetic compounds may also be useful. The term 'peptidomimetic' refers to a compound that mimics the conformation

and desirable features of a particular peptide as a therapeutic agent.

[0074] For example, the said polypeptide includes not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Meziere et al. (1997) J. Immunol. 159, 3230-3237. This approach involves making pseudo-peptides containing changes involving the backbone, and not the orientation of side chains. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis. Alternatively, the said polypeptide may be a peptidomimetic compound wherein one or more of the amino acid residues are linked by a -y(CH_2NH)- bond in place of the conventional amide linkage.

[0075] In a further alternative, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the carbon atoms of the amino acid residues is used; it may be advantageous for the linker moiety to have substantially the same charge distribution and substantially the same planarity as a peptide bond.

[0076] It will also be appreciated that the said polypeptide may conveniently be blocked at its Nor C-terminus so as to help reduce susceptibility to exo-proteolytic digestion.

[0077] A variety of un-coded or modified amino acids such as D-amino acids and N-methyl amino acids have also been used to modify mammalian peptides. In addition, a presumed bioactive conformation may be stabilised by a covalent modification, such as cyclisation or by incorporation of lactam or other types of bridges, for example see Veber et al., 1978, Proc. Natl. Acad. Sci. USA 75:2636 and Thursell et al., 1983, Biochem. Biophys. Res. Comm. 111:166.

[0078] In one embodiment, the bispecific antibody of the invention is capable of inducing tumour immunity. This can be tested *in vitro* in T cell activation assays, *e.g.* by measuring IL-2 and IFNy production. Activation of effector T cells would indicate that a tumour specific T cell response can be achieved *in vivo.* Further, an anti-tumour response in an *in vivo* model, such as a mouse model would imply that a successful immune response towards the tumour has been achieved.

[0079] Thus, the bispecific antibody may modulate the activity of a target immune system cell, wherein said modulation is an increase or decrease in the activity of said cell. Such cells include T cells, dendritic cells and natural killer cells.

[0080] The immune system cell is typically a T cell. Thus, the antibody may increase the activity of a CD4+ or CD8+ effector T cell, or may decrease the activity of a regulatory T cell (Treg). In either case, the net effect of the antibody will be an increase in the activity of effector T cells, particularly CD8+ effector T cells. Methods for determining a change in the activity of effector T cells are well known and include, for example, measuring for an increase in the level of T cell cytokine production (*e.g.* IFN-γ or IL-2) or an increase in T cell proliferation in the presence of the antibody relative to the level of T cell cytokine production and/or T cell proliferation in the presence of a control. Assays for cell proliferation and/or cytokine production are well known.

[0081] For example, the antibody may be capable of inducing:

1. (a) activation of cytotoxic T cells, *i.e.* CD8+ T cells;
2. (b) activation of helper T cells, *i.e.* $CD4^+$ T cells;
3. (c) activation of dendritic cells;
4. (d) activation of natural killer cells; and/or
5. (e) reprograming of Tregs into effector T cells (see Akhmetzyanova et al., 2016, J Immunol. 196(1):484-92).

[0082] The antibody of the invention or binding domains thereof can also be characterised and defined by their binding abilities. Standard assays to evaluate the binding ability of ligands towards targets are well known in the art, including for example, ELISAs, Western blots, RIAs, and flow cytometry analysis. The binding kinetics (*e.g.*, binding affinity) of the polypeptide also can be assessed by standard assays known in the art, such as by Surface Plasmon Resonance analysis (SPR).

[0083] The terms "binding activity" and "binding affinity" are intended to refer to the tendency of a polypeptide molecule to bind or not to bind to a target. Binding affinity may be quantified by determining the dissociation constant (Kd) for a polypeptide and its target. A lower Kd is indicative of a higher affinity for a target. Similarly, the specificity of binding of a polypeptide to its target may be defined in terms of the comparative dissociation constants (Kd) of the polypeptide for its target as compared to the dissociation constant with respect to the polypeptide and another, non-target molecule.

[0084] The value of this dissociation constant can be determined directly by well-known methods, and can be computed even for complex mixtures by methods such as those, for example, set forth in Caceci et al. (Byte 9:340-362, 1984). For example, the Kd may be established using a double-filter nitrocellulose filter binding assay such as that disclosed by Wong & Lohman (Proc. Natl. Acad. Sci. USA 90, 5428-5432, 1993). Other standard assays to evaluate the binding ability of ligands such as antibodies towards targets are known in the art, including for example, ELISAs, Western blots, RIAs, and flow cytometry analysis. The binding kinetics (*e.g.*, binding affinity) of the antibody also can be assessed by standard assays known in the art, such as by Biacore™ system analysis.

[0085] A competitive binding assay can be conducted in which the binding of the antibody to the target is compared to the binding of the target by another, known ligand of that target, such as another antibody. The concentration at which 50% inhibition occurs is known as the Ki. Under ideal conditions, the Ki is equivalent to Kd. The Ki value will never be less than the Kd, so measurement of Ki can conveniently be substituted to provide an upper limit for Kd.

[0086] Alternative measures of binding affinity include EC50 or IC50. In this context EC50 indicates the concentration at which a polypeptide achieves 50% of its maximum binding to a fixed quantity of target. IC50 indicates the concentration at which a polypeptide inhibits 50% of the maximum binding of a fixed quantity of competitor to a fixed quantity of target. In both cases, a lower level of EC50 or IC50 indicates a higher affinity for a target. The EC50 and IC50 values of a ligand for its target can both be determined by well-known methods, for example ELISA. Suitable assays to assess the EC50 and IC50 of polypeptides are set out in the Examples.

[0087] A bispecific antibody of the invention is preferably capable of binding to its target with an affinity that is at least two-fold, 10-fold, 50-fold, 100-fold or greater than its affinity for binding to another non-target molecule.

CD137 binding domains

[0088] The bispecific antibodies of the invention comprise a binding domain (B1) which is capable of specifically binding to CD137.

[0089] Advantageously, binding domain B1 binds to human CD137 with a K_D of less than 10×10^{-9}M, for example less than 4×10^{-9}M or less than 1.2×10^{-9}M.

[0090] Binding domain B1 comprises CDRL1 corresponding to SEQ ID NO: 54, CDRL2 corresponding to SEQ ID NO: 55, and CDRL3 corresponding to SEQ ID NO: 83, and CDRH1 corresponding to SEQ ID NO: 62 or a variant of CDRH1 comprising having only one amino acid mutation relative to SEQ ID NO: 62, CDRH2 corresponding to SEQ ID NO: 69, and CDRH3 corresponding to SEQ ID NO: 76.

[0091] Thus, binding domain B1 may comprisethe heavy chain variable region and/or the light chain variable region of antibody 1618/1619 (SEQ ID NO: 35 and/or SEQ ID NO: 33.

[0092] It will be appreciated by persons skilled in the art that the bispecific polypeptides of the invention may alternatively comprise variants of the above-defined variable regions.

[0093] A variant of any one of the heavy or light chain amino acid sequences recited herein may be a substitution, deletion or addition variant of said sequence. A variant may comprise 1, 2, 3, 4, 5, up to 10, up to 20, up to 30 or more amino acid substitutions and/or deletions from the said sequence. "Deletion" variants may comprise the deletion of individual amino acids, deletion of small groups of amino acids such as 2, 3, 4 or 5 amino acids, or deletion of larger amino acid regions, such as the deletion of specific amino acid domains or other features. "Substitution" variants preferably involve the replacement of one or more amino acids with the same number of amino acids and making conservative amino acid substitutions. For example, an amino acid may be substituted with an alternative amino acid having similar properties, for example, another basic amino acid, another acidic amino acid, another neutral amino acid, another charged amino acid, another hydrophilic amino acid, another hydrophobic amino acid, another polar amino acid, another aromatic amino acid or another aliphatic amino acid. Some properties of the 20 main amino acids which can be used to select suitable substituents are as follows:

Ala, A	aliphatic, hydrophobic, neutral	Met, M	hydrophobic, neutral
Cys, C	polar, hydrophobic, neutral	Asn, N	polar, hydrophilic, neutral
Asp, D	polar, hydrophilic, charged (-)	Pro, P	hydrophobic, neutral
Glu, E	polar, hydrophilic, charged (-)	Gln, Q	polar, hydrophilic, neutral
Phe, F	aromatic, hydrophobic, neutral	Arg, R	polar, hydrophilic, charged (+)

Gly, G	aliphatic, neutral	Ser, S	polar, hydrophilic, neutral
His, H	aromatic, polar, hydrophilic, charged (+)	Thr, T	polar, hydrophilic, neutral
Ile, I	aliphatic, hydrophobic, neutral	Val, V	aliphatic, hydrophobic, neutral
Lys, K	polar, hydrophilic, charged(+)	Trp, W	aromatic, hydrophobic, neutral
Leu, L	aliphatic, hydrophobic, neutral	Tyr, Y	aromatic, polar, hydrophobic

[0094] Amino acids herein may be referred to by full name, three letter code or single letter code.

[0095] Preferred "derivatives" or "variants" include those in which instead of the naturally occurring amino acid the amino acid which appears in the sequence is a structural analog thereof. Amino acids used in the sequences may also be derivatised or modified, e.g. labelled, providing the function of the antibody is not significantly adversely affected.

[0096] Derivatives and variants as described above may be prepared during synthesis of the antibody or by post- production modification, or when the antibody is in recombinant form using the known techniques of site- directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids.

[0097] Preferably variants have an amino acid sequence which has more than 60%, or more than 70%, e.g. 75 or 80%, preferably more than 85%, e.g. more than 90 or 95% amino acid identity to a sequence as shown in the sequences disclosed herein (e.g. the VH or VL region sequences, or CDR sequences therein). This level of amino acid identity may be seen across the full length of the relevant SEQ ID NO sequence or over a part of the sequence, such as across 20, 30, 50, 75, 100, 150, 200 or more amino acids, depending on the size of the full-length polypeptide.

[0098] For example, variants of the CDRH1 sequence have only one amino acid mutation relative to the reference sequence (such as a deletion, substitution and/or insertion of an amino acid).

[0099] In connection with amino acid sequences, "sequence identity" refers to sequences which have the stated value when assessed using ClustalW (Thompson *etal.,* 1994, *Nucleic Acids* Res. **22**(22):4673-80; the disclosures of which are incorporated herein by reference) with the following parameters:
Pairwise alignment parameters -Method: accurate, Matrix: PAM, Gap open penalty: 10.00, Gap extension penalty: 0.10;

[0100] Multiple alignment parameters -Matrix: PAM, Gap open penalty: 10.00, % identity for delay: 30, Penalize end gaps: on, Gap separation distance: 0, Negative matrix: no, Gap extension penalty: 0.20, Residue-specific gap penalties: on, Hydrophilic gap penalties: on, Hydrophilic residues: GPSNDQEKR. Sequence identity at a particular residue is intended to include identical residues which have simply been derivatised.

[0101] Thus, in one embodiment binding domain B1 may comprises one or more variants of the

above-defined light chain variable regions and/or said heavy chain variable regions having at least 90% sequence identity thereto.

[0102] In preferred embodiments, binding domain B1 comprises the heavy chain and/or the light chain of antibody 1618/1619.

[0103] Alternatively, binding domain B1 comprises the light chain variable region and the heavy chain variable region of antibody 1618/1619 (SEQ ID NO: 35 and/or SEQ ID NO: 33), or a variant which has more than 60%, or more than 70%, *e.g.* 75 or 80%, preferably more than 85%, e.g. more than 90 or 95% amino acid identity to SEQ ID NO: 35 and/or SEQ ID NO: 33).

Tumour cell-targeting domains

[0104] The bispecific polypeptides of the invention further comprise a binding domain (B2) which is capable of specifically binding a tumour cell-associated antigen, wherein the tumour cell-associated antigen is 5T4.

[0105] By "tumour cell-associated antigen" we include proteins accessible on the extracellular surface of tumour cells, such that they are accessible to the bispecific polypeptides of the invention following administration into the body. In one embodiment, the tumour cell-associated antigen is tumour specific, *i.e.* it is found exclusively on tumour cells and not on normal, healthy cells. However, it will be appreciated by persons skilled in the art that the tumour cell-associated antigen may be preferentially expressed on tumour cells, *i.e.* it is expressed on tumour cells at a higher level than on normal, healthy cells (thus, expression of the antigen on tumour cells may be at least five times more than on normal, healthy cells, for example expression levels on tumour cells of at least ten times more, twenty times more, fifty time more or greater).

[0106] The tumour cell-associated antigen is the oncofetal antigen, 5T4 (for example, see UniProt Q13641).

[0107] In one embodiment, the tumour cell is a solid tumour cell.

[0108] For example, the solid tumour may be selected from the groups consisting of renal cell carcinoma, colorectal cancer, lung cancer, prostate cancer, breast cancer, melanomas, bladder cancer, brain/CNS cancer, cervical cancer, oesophageal cancer, gastric cancer, head/neck cancer, kidney cancer, liver cancer, lymphomas, ovarian cancer, pancreatic cancer and sarcomas.

[0109] Advantageously, binding domain B2 binds to the tumour cell-associated antigen with a K_D of less than 10 $\times 10^{-9}$M, for example less than 4 $\times 10^{-9}$M or less than 1.2$\times 10^{-9}$M.

[0110] Binding domain B2 comprises CDRL1 corresponding to SEQ ID NO: 54 or a variant of CDRL1 having only one amino acid mutation relative to SEQ ID NO: 54, CDRL2 corresponding to SEQ ID NO: 55, and CDRL3 corresponding to SEQ ID NO: 58; and CDRH1 corresponding to SEQ ID NO: 46, CDRH2 corresponding to SEQ ID NO: 48, and CDRH3 corresponding to SEQ ID NO: 52.

[0111] Thus, binding domain B2 may comprise the heavy chain variable region and/or the light chain variable region of antibody 1210/1211 (SEQ ID NO: 11 and/or SEQ ID NO: 9).

[0112] It will be appreciated by skilled persons that binding domain B2 may alternatively comprise variants of said light chain variable regions and/or said heavy chain variable regions, for example having at least 90% sequence identity thereto.

[0113] For example, variants of the CDRL1 sequence have only one amino acid mutation relative to the reference sequence (such as a deletion, substitution and/or insertion of an amino acid).

[0114] In one embodiment, binding domain B2 comprises the heavy chain and/or the light chain of antibody 1210/1211.

[0115] Alternatively, binding domain B2 comprises the heavy chain variable region and the light chain variable region of antibody 1210/1211 (SEQ ID NO: 11 and SEQ ID NO: 9), or a variant which has more than 60%, or more than 70%, *e.g.* 75 or 80%, preferably more than 85%, e.g. more than 90 or 95% amino acid identity to SEQ ID NO: 11 and/or SEQ ID NO: 9).

Exemplary CD137- 5T4 bispecific antibodies

[0116] In one preferred embodiment of the bispecific polypeptides of the invention, binding domain B1 is an IgG and binding domain B2 is an scFv. Conversely, binding domain B1 may be an scFv and binding domain B2 may be an IgG.

[0117] In an alternative embodiment of the bispecific polypeptides of the invention, binding domain B1 is an scFv and binding domain B2 is an scFv (*e.g.* in an $scFv_2$-Fc format).

[0118] In exemplary bispecific polypeptides of the invention, B1 comprises the three CDRs of the light chain and the three CDRs of the heavy chain of antibody 1618/1619 (SEQ ID NOs: 54, 55 and 83 and SEQ ID NOs: 62, 69 and 76) and B2 comprises the three CDRs of the light chain and the three CDRs of the heavy chain of antibody 1210/1211 (SEQ ID NOs: 54, 55 and 58 and SEQ ID NOs: 46, 48 and 52).

[0119] In a preferred embodiment, B1 comprises the light chain variable region and the heavy chain variable region of antibody 1210/1211 (SEQ ID NO: 11 and/or SEQ ID NO: 9) and B2 comprises the light chain variable region and the heavy chain variable region of antibody 1618/1619 (SEQ ID NO: 35 and/or SEQ ID NO: 33), or variants of said light chain variable regions and/or said heavy chain variable regions (for example, having at least 90% sequence identity thereto).

[0120] Typically, the bispecific antibody polypeptides of the invention will comprise constant region sequences, in addition to the above-defined variable region sequences.

[0121] An exemplary heavy chain constant region amino acid sequence which may be combined with any VH region sequence disclosed herein (to form a complete heavy chain) is the following IgG1 heavy chain constant region sequence:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[SEQ ID NO:94]

or a variant thereof comprising the L234A and L235A ("LALA") mutations (see amino acid residues highlighted above).

[0122] Likewise, an exemplary light chain constant region amino acid sequence which may be combined with any VL region sequence disclosed herein (to form a complete light chain) is the kappa chain constant region sequence reproduced here:
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[SEQ ID NO:95].

[0123] Thus, the bispecific antibody of the invention may comprise:

1. (a) a binding domain (B1) comprising a heavy chain variable region of any of SEQ ID NOs: 33 and a light chain variable region of any of SEQ ID NOs: 35;
2. (b) a heavy chain constant region comprising an Fc region (for example, SEQ ID NO: 94 or 96);
3. (c) a binding domain (B2) comprising a heavy chain variable region of any of SEQ ID NOs: 9 and a light chain variable region of any of SEQ ID NOs: 11; and
4. (d) optionally, a light chain constant region (for example SEQ ID NO:95).

[0124] In one preferred embodiment, the bispecific antibody of the invention is an IgG-scFv bispecific antibody (for example, wherein B1 is an intact IgG and B2 is an scFv attached to the C-terminus of a heavy chain of the IgG, or *vice versa*).

[0125] For example, the bispecific antibody may comprise the following components:

1. (a) two heavy chains each comprising, in order from the N-terminus to the C terminus:
   [a VH sequence] - [an H chain constant region] - [a connector] - [an scFv]
   wherein the scFv may comprise of consist of in order from the N-terminus to the C terminus:
   [a VH sequence] - [a linker] - [a VL sequence], or *vice versa*
2. (b) two light chains each comprising, in order from the N-terminus to the C terminus:
   [a VL sequence] - [an L chain constant region]

[0126] In such "Morrison format" bispecific antibodies:

- the VH sequences may be selected from clone 1618 (SEQ ID NO: 33), clone 1210 (SEQ ID

NO:9) or a variant thereof;
- the H chain constant region may be selected from any of those disclosed herein, for example SEQ ID NO:86 or 94;
- the connector may be selected from any of those disclosed herein, for example SEQ ID NOs:92 or 140 or 143;
- the linker within the scFv may be selected from any of those disclosed herein, for example SEQ ID NO:93, and
- the VL sequence within the scFv may be selected from clone 1619 (SEQ ID NO: 35), clone 1211 (SEQ ID NO: 11) or a variant thereof; and
- the L chain constant region may be selected from any of those disclosed herein, for example SEQ ID NO:95.

[0127] As discussed above, methods for the production of antibody polypeptides of the invention are well known in the art.

[0128] Conveniently, the antibody polypeptide is or comprises a recombinant polypeptide. Suitable methods for the production of such recombinant polypeptides are well known in the art, such as expression in prokaryotic or eukaryotic hosts cells (for example, see Green & Sambrook, 2012, Molecular Cloning, A Laboratory Manual, Fourth Edition, Cold Spring Harbor, New York).

[0129] Antibody polypeptides of the invention can also be produced using a commercially available *in vitro* translation system, such as rabbit reticulocyte lysate or wheatgerm lysate (available from Promega). Preferably, the translation system is rabbit reticulocyte lysate. Conveniently, the translation system may be coupled to a transcription system, such as the TNT transcription-translation system (Promega). This system has the advantage of producing suitable mRNA transcript from an encoding DNA polynucleotide in the same reaction as the translation.

[0130] It will be appreciated by persons skilled in the art that antibody polypeptides of the invention may alternatively be synthesised artificially, for example using well known liquid-phase or solid phase synthesis techniques (such as *t*-Boc or Fmoc solid-phase peptide synthesis).

Polynucleotides, vectors and cells

[0131] A second aspect of the invention provides an isolated nucleic acid molecule encoding a bispecific polypeptide according to any one of the bispecific antibodies as described in the claims.

[0132] The terms "nucleic acid molecule" and "polynucleotide" are used interchangeably herein and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Non-limiting examples of polynucleotides include a gene, a gene fragment, messenger RNA (mRNA), cDNA, recombinant polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide of the invention may be provided in isolated or substantially isolated form. By substantially isolated, it is meant that there may be substantial, but not total, isolation of the

polypeptide from any surrounding medium. The polynucleotides may be mixed with carriers or diluents which will not interfere with their intended use and still be regarded as substantially isolated.

[0133] A nucleic acid sequence which "encodes" a selected polypeptide is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) 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' (carboxy) terminus. For the purposes of the invention, such nucleic acid sequences can include, but are not limited to, cDNA from viral, prokaryotic or eukaryotic mRNA, genomic sequences from viral or prokaryotic DNA or RNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence.

[0134] A suitable polynucleotide sequence may alternatively be a variant. For example, a variant may be a substitution, deletion or addition variant of any of the above nucleic acid sequences. A variant polynucleotide may comprise 1, 2, 3, 4, 5, up to 10, up to 20, up to 30, up to 40, up to 50, up to 75 or more nucleic acid substitutions and/or deletions from the sequences given in the sequence listing.

[0135] Variants may be at least 70% homologous to a polynucleotide of any one of nucleic acid sequences disclosed herein, preferably at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto. Preferably homology and identity at these levels is present at least with respect to the coding regions of the polynucleotides. Methods of measuring homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is calculated on the basis of nucleic acid identity. Such homology may exist over a region of at least 15, preferably at least 30, for instance at least 40, 60, 100, 200 or more contiguous nucleotides. Such homology may exist over the entire length of the unmodified polynucleotide sequence.

[0136] Methods of measuring polynucleotide homology or identity are known in the art. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (e.g. used on its default settings) (Devereux et al, 1984, Nucleic Acids Research 12:387-395).

[0137] The PILEUP and BLAST algorithms can also be used to calculate homology or line up sequences (typically on their default settings), for example as described in Altschul, 1993, J Mol Evol 36:290-300; Altschul et al, 1990, J Mol Biol 215:403-10).

[0138] Software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al, supra*). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is

reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, 1992, Proc. Natl. Acad. Sci. USA 89:10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

[0139] The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see *e.g.* Karlin & Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[0140] The homologue may differ from a sequence in the relevant polynucleotide by less than 3, 5, 10, 15, 20 or more mutations (each of which may be a substitution, deletion or insertion).

[0141] These mutations may be measured over a region of at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides of the homologue.

[0142] In one embodiment, a variant sequence may vary from the specific sequences given in the sequence listing by virtue of the redundancy in the genetic code. The DNA code has 4 primary nucleic acid residues (A, T, C and G) and uses these to "spell" three letter codons which represent the amino acids the proteins encoded in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons. A variant polynucleotide of the invention may therefore encode the same polypeptide sequence as another polynucleotide of the invention, but may have a different nucleic acid sequence due to the use of different codons to encode the same amino acids.

[0143] A bispecific antibody of the invention may thus be produced from or delivered in the form of a polynucleotide which encodes, and is capable of expressing, it.

[0144] Polynucleotides of the invention can be synthesised according to methods well known in the art, as described by way of example in Green & Sambrook (2012, Molecular Cloning - a laboratory manual, 4th edition; Cold Spring Harbor Press).

[0145] The nucleic acid molecules of the present invention may be provided in the form of an expression cassette which includes control sequences operably linked to the inserted sequence, thus allowing for expression of the polypeptide of the invention *in vivo.* These expression cassettes, in turn, are typically provided within vectors (e.g., plasmids or recombinant viral vectors). Such an expression cassette may be administered directly to a host subject. Alternatively, a vector comprising a polynucleotide of the invention may be administered to a host subject. Preferably the polynucleotide is prepared and/or administered using a genetic vector. A suitable vector may be any

vector which is capable of carrying a sufficient amount of genetic information, and allowing expression of an antibody of the invention.

[0146] The present invention thus includes expression vectors that comprise such polynucleotide sequences. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for expression of a peptide of the invention. Other suitable vectors would be apparent to persons skilled in the art (see Green & Sambrook, *supra*).

[0147] The invention also includes cells that have been modified to express a bispecific antibody of the invention. Such cells include transient, or preferably stable higher eukaryotic cell lines, such as mammalian cells or insect cells, lower eukaryotic cells, such as yeast or prokaryotic cells such as bacterial cells. Particular examples of cells which may be modified by insertion of vectors or expression cassettes encoding for a polypeptide of the invention include mammalian HEK293T, CHO, HeLa, NS0 and COS cells. Preferably the cell line selected will be one which is not only stable, but also allows for mature glycosylation and cell surface expression of a polypeptide.

[0148] Such cell lines of the invention may be cultured using routine methods to produce a bispecific antibody of the invention, or may be used therapeutically or prophylactically to deliver antibodies of the invention to a subject. Alternatively, polynucleotides, expression cassettes or vectors of the invention may be administered to a cell from a subject *ex vivo* and the cell then returned to the body of the subject.

[0149] In one embodiment, the nucleic acid molecule encodes an antibody heavy chain or variable region thereof.

[0150] In one embodiment, the nucleic acid molecule encodes an antibody light chain or variable region thereof.

[0151] By "nucleic acid molecule" we include DNA (*e.g.* genomic DNA or complementary DNA) and mRNA molecules, which may be single- or double-stranded. By "isolated" we mean that the nucleic acid molecule is not located or otherwise provided within a cell.

[0152] In one embodiment, the nucleic acid molecule is a cDNA molecule.

[0153] It will be appreciated by persons skilled in the art that the nucleic acid molecule may be codon-optimised for expression of the antibody polypeptide in a particular host cell, *e.g.* for expression in human cells (for example, see Angov, 2011, Biotechnol. J. 6(6):650-659).

[0154] Also included within the scope of the invention are the following:

1. (a) a third aspect of the invention provides a vector (such as an expression vector) comprising a nucleic acid molecule according to the second aspect of the invention;
2. (b) a fourth aspect of the invention provides a recombinant host cell (such as a mammalian cell, *e.g.* human cell, or Chinese hamster ovary cell, *e.g.* CHOK1SV cells) comprising a

nucleic acid molecule according to the second aspect of the invention or a vector according to the third aspect of the invention; and

3. (c) a fifth aspect of the invention provides a method of making a bispecific antibody according to the first aspect of the invention comprising culturing a population of host cells according to the fourth aspect of the invention under conditions in which said polypeptide is expressed, and isolating the bispecific antibody therefrom.

[0155] In a sixth aspect, the present invention provides pharmaceutical compositions comprising an effective amount of bispecific antibody of the invention. The invention provides a pharmaceutical composition further comprising at least one pharmaceutically acceptable diluent, carrier or excipient.

[0156] It will be appreciated by persons skilled in the art that additional compounds may also be included in the pharmaceutical compositions, including, chelating agents such as EDTA, citrate, EGTA or glutathione.

[0157] The pharmaceutical compositions may be prepared in a manner known in the art that is sufficiently storage stable and suitable for administration to humans and animals. For example, the pharmaceutical compositions may be lyophilised, e.g. through freeze drying, spray drying, spray cooling, or through use of particle formation from supercritical particle formation.

[0158] By "pharmaceutically acceptable" we mean a non-toxic material that does not decrease the effectiveness of the CD137 and 5T4-binding activity of the antibody polypeptide of the invention. Such pharmaceutically acceptable buffers, carriers or excipients are well-known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A.R Gennaro, Ed., Mack Publishing Company (1990) and handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed ., Pharmaceutical Press (2000)).

[0159] The term "buffer" is intended to mean an aqueous solution containing an acid-base mixture with the purpose of stabilising pH. Examples of buffers are Trizma, Bicine, Tricine, MOPS, MOPSO, MOBS, Tris, Hepes, HEPBS, MES, phosphate, carbonate, acetate, citrate, glycolate, lactate, borate, ACES, ADA, tartrate, AMP, AMPD, AMPSO, BES, CABS, cacodylate, CHES, DIPSO, EPPS, ethanolamine, glycine, HEPPSO, imidazole, imidazolelactic acid, PIPES, SSC, SSPE, POPSO, TAPS, TABS, TAPSO and TES.

[0160] The term "diluent" is intended to mean an aqueous or non-aqueous solution with the purpose of diluting the antibody polypeptide in the pharmaceutical preparation. The diluent may be one or more of saline, water, polyethylene glycol, propylene glycol, ethanol or oils (such as safflower oil, corn oil, peanut oil, cottonseed oil or sesame oil).

[0161] The term "adjuvant" is intended to mean any compound added to the formulation to increase the biological effect of the antibody polypeptide of the invention. The adjuvant may be one or more of zinc, copper or silver salts with different anions, for example, but not limited to fluoride, chloride, bromide, iodide, tiocyanate, sulfite, hydroxide, phosphate, carbonate, lactate, glycolate, citrate, borate, tartrate, and acetates of different acyl composition. The adjuvant may also be cationic polymers such as cationic cellulose ethers, cationic cellulose esters, deacetylated hyaluronic acid,

chitosan, cationic dendrimers, cationic synthetic polymers such as poly(vinyl imidazole), and cationic polypeptides such as polyhistidine, polylysine, polyarginine, and peptides containing these amino acids.

[0162] The excipient may be one or more of carbohydrates, polymers, lipids and minerals. Examples of carbohydrates include lactose, glucose, sucrose, mannitol, and cyclodextrines, which are added to the composition, *e.g.* for facilitating lyophilisation. Examples of polymers are starch, cellulose ethers, cellulose carboxymethylcellulose, hydroxypropylmethyl cellulose, hydroxyethyl cellulose, ethylhydroxyethyl cellulose, alginates, carageenans, hyaluronic acid and derivatives thereof, polyacrylic acid, polysulphonate, polyethylenglycol/polyethylene oxide, polyethyleneoxide/polypropylene oxide copolymers, polyvinylalcohol/polyvinylacetate of different degree of hydrolysis, and polyvinylpyrrolidone, all of different molecular weight, which are added to the composition, e.g., for viscosity control, for achieving bioadhesion, or for protecting the lipid from chemical and proteolytic degradation. Examples of lipids are fatty acids, phospholipids, mono-, di-, and triglycerides, ceramides, sphingolipids and glycolipids, all of different acyl chain length and saturation, egg lecithin, soy lecithin, hydrogenated egg and soy lecithin, which are added to the composition for reasons similar to those for polymers. Examples of minerals are talc, magnesium oxide, zinc oxide and titanium oxide, which are added to the composition to obtain benefits such as reduction of liquid accumulation or advantageous pigment properties.

[0163] The bispecific antibodies of the invention may be formulated into any type of pharmaceutical composition known in the art to be suitable for the delivery thereof.

[0164] In one embodiment, the pharmaceutical compositions of the invention may be in the form of a liposome, in which the antibody is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids, which exist in aggregated forms as micelles, insoluble monolayers and liquid crystals. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Suitable lipids also include the lipids above modified by poly(ethylene glycol) in the polar headgroup for prolonging bloodstream circulation time. Preparation of such liposomal formulations is can be found in for example US 4,235,871.

[0165] The pharmaceutical compositions of the invention may also be in the form of biodegradable microspheres. Aliphatic polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), copolymers of PLA and PGA (PLGA) or poly(caprolactone) (PCL), and polyanhydrides have been widely used as biodegradable polymers in the production of microspheres. Preparations of such microspheres can be found in US 5,851,451 and in EP 0 213 303.

[0166] In a further embodiment, the pharmaceutical compositions of the invention are provided in the form of polymer gels, where polymers such as starch, cellulose ethers, cellulose carboxymethylcellulose, hydroxypropylmethyl cellulose, hydroxyethyl cellulose, ethylhydroxyethyl cellulose, alginates, carageenans, hyaluronic acid and derivatives thereof, polyacrylic acid, polyvinyl imidazole, polysulphonate, polyethylenglycol/ polyethylene oxide, polyethyleneoxide/polypropylene oxide copolymers, polyvinylalcohol/ polyvinylacetate of different degree of hydrolysis, and polyvinylpyrrolidone are used for thickening of the solution containing the agent. The polymers may also comprise gelatin or collagen.

[0167] Alternatively, the antibody polypeptide may simply be dissolved in saline, water, polyethylene glycol, propylene glycol, ethanol or oils (such as safflower oil, corn oil, peanut oil, cottonseed oil or sesame oil), tragacanth gum, and/or various buffers.

[0168] It will be appreciated that the pharmaceutical compositions of the invention may include ions and a defined pH for potentiation of action of the active antibody polypeptide. Additionally, the compositions may be subjected to conventional pharmaceutical operations such as sterilisation and/or may contain conventional adjuvants such as preservatives, stabilisers, wetting agents, emulsifiers, buffers, fillers, *etc.*

[0169] The pharmaceutical compositions according to the invention may be administered via any suitable route known to those skilled in the art. Thus, possible routes of administration include parenteral (intravenous, subcutaneous, and intramuscular), topical, ocular, nasal, pulmonar, buccal, oral, parenteral, vaginal and rectal. Also administration from implants is possible.

[0170] In one preferred embodiment, the pharmaceutical compositions are administered parenterally, for example, intravenously, intracerebroventricularly, intraarticularly, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intramuscularly or subcutaneously, or they may be administered by infusion techniques. They are conveniently used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.

[0171] Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

[0172] Thus, the pharmaceutical compositions of the invention are particularly suitable for parenteral, *e.g.* intravenous, administration.

[0173] Alternatively, the pharmaceutical compositions may be administered intranasally or by inhalation (for example, in the form of an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, such as dichlorodifluoromethane, trichlorofluoro-methane, dichlorotetrafluoro-ethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A3 or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other suitable gas). In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active polypeptide, *e.g.* using a mixture of ethanol and

the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

[0174] The pharmaceutical compositions will be administered to a patient in a pharmaceutically effective dose. A 'therapeutically effective amount', or 'effective amount', or 'therapeutically effective', as used herein, refers to that amount which provides a therapeutic effect for a given condition and administration regimen. This is a predetermined quantity of active material calculated to produce a desired therapeutic effect in association with the required additive and diluent, *i.e*. a carrier or administration vehicle. Further, it is intended to mean an amount sufficient to reduce and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in a host. As is appreciated by those skilled in the art, the amount of a compound may vary depending on its specific activity. Suitable dosage amounts may contain a predetermined quantity of active composition calculated to produce the desired therapeutic effect in association with the required diluent. In the methods and use for manufacture of compositions of the invention, a therapeutically effective amount of the active component is provided. A therapeutically effective amount can be determined by the ordinary skilled medical or veterinary worker based on patient characteristics, such as age, weight, sex, condition, complications, other diseases, *etc.,* as is well known in the art. The administration of the pharmaceutically effective dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administrations of subdivided doses at specific intervals. Alternatively, the does may be provided as a continuous infusion over a prolonged period.

[0175] Particularly preferred compositions are formulated for systemic administration.

[0176] The composition may preferably be formulated for sustained release over a period of time. Thus the composition may be provided in or as part of a matrix facilitating sustained release. Preferred sustained release matrices may comprise a montanide or γ-polyglutamic acid (PGA) nanoparticles.

[0177] The antibodies can be formulated at various concentrations, depending on the efficacy/toxicity of the polypeptide being used. For example, the formulation may comprise the active antibody at a concentration of between 0.1 μM and 1 mM, more preferably between 1 μM and 500 μM, between 500 μM and 1 mM, between 300 μM and 700 μM, between 1 μM and 100 μM, between 100 μM and 200 μM, between 200 μM and 300 μM, between 300 μM and 400 μM, between 400 μM and 500 μM, between 500 μM and 600 μM, between 600 μM and 700 μM, between 800 μM and 900 μM or between 900 μM and 1 mM. Typically, the formulation comprises the active antibody at a concentration of between 300 μM and 700 μM.

[0178] Typically, the therapeutic dose of the antibody (with or without a therapeutic moiety) in a human patient will be in the range of 100 μg to 700 mg per administration (based on a body weight of 70kg). For example, the maximum therapeutic dose may be in the range of 0.1 to 10 mg/kg per administration, *e.g*. between 0.1 and 5 mg/kg or between 1 and 5 mg/kg or between 0.1 and 2 mg/kg. It will be appreciated that such a dose may be administered at different intervals, as

determined by the oncologist/physician; for example, a dose may be administered daily, twice-weekly, weekly, bi-weekly or monthly.

[0179] It will be appreciated by persons skilled in the art that the pharmaceutical compositions of the invention may be administered alone or in combination with other therapeutic agents used in the treatment of cancers, such as antimetabolites, alkylating agents, anthracyclines and other cytotoxic antibiotics, vinca alkyloids, etoposide, platinum compounds, taxanes, topoisomerase I inhibitors, other cytostatic drugs, antiproliferative immunosuppressants, corticosteroids, sex hormones and hormone antagonists, and other therapeutic antibodies (such as antibodies against a tumour-associated antigen or an immune checkpoint modulator).

[0180] For example, the pharmaceutical compositions of the invention may be administered in combination with an immunotherapeutic agent that binds a target selected from the group consisting of PD-1/PD-1L, CTLA-4, OX40, CD40, GITR, LAG3, TIM3, CD27 and KIR.

[0181] Thus, the invention encompasses combination therapies comprising a bispecific antibody of the invention together with a further immunotherapeutic agent, effective in the treatment of cancer, which specifically binds to an immune checkpoint molecule. It will be appreciated that the therapeutic benefit of the further immunotherapeutic agent may be mediated by attenuating the function of an inhibitory immune checkpoint molecule and/or by activating the function of a stimulatory immune checkpoint or co-stimulatory molecule.

[0182] In one embodiment, the further immunotherapeutic agent is selected from the group consisting of:

1. (a) an immunotherapeutic agent that inhibits the function of PD-1 and/or PD-1L;
2. (b) an immunotherapeutic agent that inhibits the function of CTLA-4;
3. (c) an immunotherapeutic agent that activates the function of OX40; and
4. (d) an immunotherapeutic agent that binds activates the function of CD40.

[0183] Thus, the further immunotherapeutic agent may be a PD1 inhibitor, such as an anti-PD1 antibody, or antigen-binding fragment thereof capable of inhibiting PD1 function (for example, Nivolumab, Pembrolizumab, Lambrolizumab, PDR-001, MEDI-0680 and AMP-224). Alternatively, the PD1 inhibitor may comprise or consist of an anti-PD-L1 antibody, or antigen-binding fragment thereof capable of inhibiting PD1 function (for example, Durvalumab, Atezolizumab, Avelumab and MDX-1105).

[0184] In another embodiment, the further immunotherapeutic agent is a CTLA-4 inhibitor, such as an anti-CTLA-4 antibody or antigen-binding portion thereof.

[0185] In a further embodiment, the further immunotherapeutic agent activates OX40, such as an agonistic anti-OX40 antibody or antigen-binding portion thereof.

[0186] In a further embodiment, the further immunotherapeutic agent activates CD40, such as an agonistic anti-CD40 antibody or antigen-binding portion thereof.

[0187] It will be appreciated by persons skilled in the art that the presence of the two active agents (as detailed above) may provide a synergistic benefit in the treatment of a tumour in a subject. By "synergistic" we include that the therapeutic effect of the two agents in combination (*e.g.* as determined by reference to the rate of growth or the size of the tumour) is greater than the additive therapeutic effect of the two agents administered on their own.

[0188] Such synergism can be identified by testing the active agents, alone and in combination, in a relevant cell line model of the solid tumour.

Medical uses and methods

[0189] The antibodies in accordance with the present invention may be used in therapy or prophylaxis. In therapeutic applications, antibodies or compositions are administered to a subject already suffering from a disorder or condition, in an amount sufficient to cure, alleviate or partially arrest the condition or one or more of its symptoms. Such therapeutic treatment may result in a decrease in severity of disease symptoms, or an increase in frequency or duration of symptom-free periods. An amount adequate to accomplish this is defined as "therapeutically effective amount". In prophylactic applications, antibodies or compositions are administered to a subject not yet exhibiting symptoms of a disorder or condition, in an amount sufficient to prevent or delay the development of symptoms. Such an amount is defined as a "prophylactically effective amount". The subject may have been identified as being at risk of developing the disease or condition by any suitable means.

[0190] Thus, a seventh aspect of the invention provides a bispecific antibody according to the first aspect of the invention for use in medicine.

[0191] An eighth aspect of the invention provides a bispecific antibody according to the first aspect of the invention for use in treating a neoplastic disorder in a subject.

[0192] By 'treatment' we include both therapeutic and prophylactic treatment of the patient. The term 'prophylactic' is used to encompass the use of an agent, or formulation thereof, as described herein which either prevents or reduces the likelihood of a neoplastic disorder, or the spread, dissemination, or metastasis of cancer cells in a patient or subject. The term 'prophylactic' also encompasses the use of an agent, or formulation thereof, as described herein to prevent recurrence of a neoplastic disorder in a patient who has previously been treated for the neoplastic disorder.

[0193] In one embodiment, the neoplastic disorder is associated with the formation of solid tumours within the subject's body.

[0194] Thus, the solid tumour may be selected from the group consisting of prostate cancer, breast cancer, lung cancer, colorectal cancer, melanomas, bladder cancer, brain/CNS cancer, cervical cancer, oesophageal cancer, gastric cancer, head/neck cancer, kidney cancer, liver cancer, lymphomas, ovarian cancer, pancreatic cancer and sarcomas.

[0195] For example, the solid tumour may be selected from the groups consisting of renal cell carcinoma, colorectal cancer, lung cancer, prostate cancer and breast cancer.

[0196] In one embodiment, the subject is human.

[0197] In one embodiment, the method comprises administering the bispecific antibody systemically.

[0198] In one embodiment, the methods further comprises administering to the subject one or more additional therapeutic agents.

[0199] The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

[0200] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[0201] These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the above description and the accompanying drawings.

[0202] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Brief description of the figures

[0203]

Figure 1 shows a schematic representation of the structure of exemplary formats for a bispecific antibody of the invention. In each format, the constant regions are shown as filled light grey; variable heavy chain regions VH1 are shown as chequered black and white; variable light chain regions VL1 are shown as filled white; variable heavy chain regions VH2 are shown as filled black; and variable light chain regions VL2 are shown as white with diagonal lines. CD137 binding domains (binding domain 1) are typically represented as a pair of a chequered black and white domain with a filled white domain (VH1/VL1); tumour-associated antigen binding domains (binding domain 2) are typically represented as a pair of a filled black domain and a white domain with diagonal lines (VH2/VL2). However, in all of the formats shown, it will be appreciated that binding domains 1 and 2 may be switched. That is, a CD137 binding domain may occur in a position shown in this figure for a tumour-associated antigen domain, and *vice versa.*

Figure 2 shows an example of a dose-response experiment of 5T4 antibodies binding to 5T4-transfected B16 cells, analysed by flow cytometry.

Figure 3 shows flow cytometry data showing normalized mean fluorescence intensity (MFI) of 5T4 mAb binding at a concentration of 2.5 µg/ml to 5T4-transfected B16 cells. The figure shows the mean ± SD of the pooled data from four experiments, with 1-4 data points for each antibody, as indicated in Table 2. MFI values were normalised to reference antibody 1628.

Figure 4 shows dose-response analysis of 5T4 antibody binding to cynomolgus 5T4-transfected CHO cells.

Figure 5 is an illustration of 5T4 chimeras used for epitope mapping of 5T4 antibodies. A: Each of the indicated domains E1-E7 were replaced by mouse 5T4 sequence in human/mouse chimeras. B: aa 173-420 were replaced by mouse 5T4 sequence

Figure 6 shows binding of exemplary anti-CD137 antibodies to human and cynomolgus CD137. Data from two separate experiments are included.

Figure 7 shows an overview of human/mouse CD137 chimeras. Black: mouse sequence, white: human sequence.

Figure 8 shows stimulation index values normalized to reference 1811/1812.

Figure 9 shows the summary of two experiments of CD137 mAb competition with CD137L binding to CHO-huCD137 cells (25 µg/ml).

Figure 10 shows CD137 activation in the presence of crosslinking antibody.

Figure 11 shows CD137 activation in the absence of crosslinking antibody.

Figure 12 shows dose-response curves in dual ELISA of 5T4-CD137 bispecific antibodies. Each graph includes data based on one 5T4 binder (1206 [i.e. 1206/1207], 1208, 1210 or 1212) combined with various CD137 agonistic antibodies (1200 [*i.e.* 1200/1201], 1202, 1204, 1214, 1618, 1620 or 1626).

Figure 13 shows 5T4-dependent T cell activation by exemplary and reference bispecific antibodies (bsAb). Each bsAb (1 µg/ml) was run in CD8 T cell assays based on 2-4 individual donors. The data is presented as mean fold change to reference (1200-1210) and error bars represent SD. The left part of the graph shows bispecific antibodies where the 5T4 scFv has been fused to CD137 IgG, *i.e.* 1200-1206 etc, whereas the right part of the graph shows bispecific antibodies where the CD137 scFv has been fused to 5T4 IgG, *i.e.* 1206-1200 etc.

Figure 14 shows the dose-response of 5T4-CD137 bsAbs showing 5T4 dependent T cell activation. Data is analysed as fold change to reference (1200-1210 at 1 µg/ml). Upper panel: CD137 agonist as IgG and 5T4 binder as scFv fused to C-terminus of IgG. Lower panel: 5T4 binder as IgG and CD137 agonist as scFv fused to C-terminus of IgG. Clone designation follows the same principle as described for Figure 10.

Figure 15 shows the functional activity of exemplary and reference 5T4-CD137 bispecific antibodies on human CD8+ T cells cultured with 5T4-expressing tumor cells. All generated bsAbs were evaluated at 1 µg/ml in the fully cell-based T cell assay to verify the results obtained in the assay performed with coated 5T4-Fc. Results are presented as fold change to reference (1200-1210) and the error bars are the SD. Clone designation follows the same principle as described for Figure 10.

Figure 16 shows binding curves for 5T4 lead optimised clones to (A) CHOh5T4 and (B) CHOcyno5T4 cells.

Figure 17 shows binding curves for CD137 lead optimised clones to (A) CHOhCD137 and (B) CHOcynoCD137 cells.

Figure 18 shows the normalised interferon gamma (IFNγ) response in human CD8+ T cells cultured in 5T4-Fc coated plates, represented as a three-parameter sigmoidal dose-response model to enable determination of EC50.

Figure 19 shows results for lead optimised bsAb in a CD8+ T cell assay with crosslinked 5T4-Fc, with normalised IFNγ levels to enable correlation of results between assay plates.

Figure 20 shows results for bsAbs generated with different linkers in a CD8+ T cell assay with crosslinked 5T4-Fc, with normalised IFNγ levels to enable correlation of results between assay plates.

Figure 21 shows the normalised interferon gamma (IFNγ) response using lead optimised bsAb in human CD8+ T cells cultured with 5T4-expressing and 5T4-non-expressing tumour cells, represented as a three-parameter sigmoidal dose-response model to enable determination of EC50

Figure 22 shows the interferon gamma (IFNγ) response from CD137-mediated activation of PBMCs with and without the presence of 5T4-Fc.

Figure 23 shows the interferon gamma (IFNγ) response from co-culture of CD8+ T cells and CD32-expressing L cells.

Figure 24 shows results from a dual ELISA detecting CD137, for TAA-CD137 bispecific antibodies.

Reference Figure 25 shows the interferon gamma (IFNγ) response using TAA-CD137 bispecific antibodies in CD8+ T cells cultured on CD3/TAA-coated plates where the TAA is (A) EpCAM, (B) EGFR and (C) Her2.

Figure 26 shows 5T4-dependent localization of bispecific antibody to the antigen-expressing tumors. B16 and B16-5T4 tumors were collected from SCID-Beige mice treated with vehicle, 1618-1210 (bsAb), 1618 (anti-CD137 Mab) or 2112 (reference anti-CD137 Mab). Localization of antibody to the tumors was detected with anti-human IgG and analyzed by flow cytometry. The graph shows the frequency of human IgG+ cells among live cells (n=5).

Figure 27 shows 5T4-dependent localization of bispecific antibody to the antigen-expressing tumors. CT26 and CT26-5T4 tumors were collected from SCID-Beige mice treated with vehicle, 1618-1210, 1618 or 2112. Localization of antibody to the tumors was detected (A) with anti-human IgG or (B) by binding of biotinylated CD137, and analyzed by flow cytometry. The graphs show the frequency of positive cells among single, live tumor cells (n=5).

Figure 28 shows the percentage of tumor cells that are positive for binding of biotinylated CD137 and the tumor antigen 5T4. SKOV-3 tumors were collected from SCID-Beige mice treated with vehicle, 1618-1210, 1618 or 2112. Localization of antibody to the 5T4 positive tumour cells was detected with anti-human IgG and anti-human 5T4-antibody. The graph show the frequency of double positive cells among single, live tumor cells (mCD45-CD45RA- (n=5/treatment).

TABLES (SEQUENCES)

Table A - VL and VH amino acid (aa) and nucleotide (nt) sequences (including claimed and reference sequences)

[0204]

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
1	1206, heavy chain, VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSGSSMSW VRQAPGKGLEWVSSIYYSGSGTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARYGRNVHPYNLDY WGQGTLVTVSS
2	1206, heavy chain, VH	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTTCTGGTTCTTCTATGTCTTG GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGG GTCTCATCTATTTACTACTCTGGTTCTGGTACATACT ATGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCC GTGACAATTCCAAGAACACGCTGTATCTGCAAATGA ACAGCCTGCGTGCCGAGGACACGGCTGTATATTATT GTGCGCGCTACGGTCGTAACGTTCATCCGTACAACT TGGACTATTGGGGCCAGGGAACCCTGGTCACCGTC TCCTCA
3	1207, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQGYYYLPTFGQGTKLEIK
4	1207, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGGGTTACTACTACCTGCCCAC TTTTGGCCAGGGGACCAAGCTGGAGATCAAA
5	1208, heavy chain VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWV RQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN
			SKNTLYLQMNSLRAEDTAVYYCARSPYYYGANWIDYW GQGTLVTVSS
6	1208, heavy	nt	

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
	chain VH		GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTAGCAGCTATGCCATGAGCT GGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG GGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATA CTATGCAGACTCCGTGAAGGGCCGGTTCACCATCTC CCGTGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGCGTGCCGAGGACACGGCTGTATATTA TTGTGCGCGCTCTCCGTACTACTACGGTGCTAACTG GATTGACTATTGGGGCCAGGGAACCCTGGTCACCGT CTCCTCA
7	1135, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQSYSTPYTFGQGTKLEIK
8	1135, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGAGTTACAGTACCCCTTATAC TTTTGGCCAGGGGACCAAGCTGGAGATCAAA
9	1210, heavy chain VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWV RQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARYYGGYYSAWMDY WGQGTLVTVSS
10	1210, heavy chain VH	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTAGCAGCTATGCCATGAGCT GGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG GGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATA CTATGCAGACTCCGTGAAGGGCCGGTTCACCATCTC CCGTGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGCGTGCCGAGGACACGGCTGTATATTA TTGTGCGCGCTACTACGGTGGTTACTACTCTGCTTG GATGGACTATTGGGGCCAGGGAACCCTGGTCACCG TCTCCTCA
11	1211, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQTYGYLHTFGQGTKLEIK
12	1211, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGACTTACGGTTACCTGCACAC

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
			TTTTGGCCAGGGGACCAAGCTGGAGATCAAA
13	1212, heavy chain VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWV RQAPGKGLEWVSYISSYGGYTSYADSVKGRFTISRDN
			SKNTLYLQMNSLRAEDTAVYYCARYHSGVLDYWGQG TLVTVSS
14	1212, heavy chain VH	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTAGCAGCTATGCCATGAGCT GGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG GGTCTCATACATTTCTTCTTACGGTGGTTACACATCT TATGCAGACTCCGTGAAGGGCCGGTTCACCATCTCC CGTGACAATTCCAAGAACACGCTGTATCTGCAAATG AACAGCCTGCGTGCCGAGGACACGGCTGTATATTAT TGTGCGCGCTACCATTCTGGTGTTTTGGACTATTGG GGCCAGGGAACCCTGGTCACCGTCTCCTCA
15	1213, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQYYYHYLLTFGQGTKLEIK
16	1213, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGTACTACTACCATTACCTGCT CACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA
17	1200, heavy chain VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWV RQAPGKGLEWVSGISGGGGGTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDVAYFDYWGQGTL VTVSS
18	1200, heavy chain VH	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTAGCAGCTATGCCATGAGCT GGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG GGTCTCAGGTATTTCTGGTGGTGGTGGTGGTACATA CTATGCAGACTCCGTGAAGGGCCGGTTCACCATCTC CCGTGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGCGTGCCGAGGACACGGCTGTATATTA TTGTGCGCGCGACGTTGCTTACTTTGACTATTGGGG CCAGGGAACCCTGGTCACCGTCTCCTCA
19	1201, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
			LQPEDFATYYCQQYYIPHTFGQGTKLEIK
20	1201, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAgCAGTCTGCAACCTGAAGAtTTTGCAAC TTATTACTGTCAACAGTACTACATTCCGCACACTTTT GGCCaGGGGACCaAGCTGGagaTCAAA
21	1202, heavy chain VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFYGSSMSW VRQAPGKGLEWVSSIYYGSSGTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARSYYGYFDYWGQ GTLVTVSS
22	1202, heavy chain VH	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTTACGGTTCTTCTATGTCTTG GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGG GTCTCATCTATTTACTACGGTTCTTCTGGTACATACT ATGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCC GTGACAATTCCAAGAACACGCTGTATCTGCAAATGA ACAGCCTGCGTGCCGAGGACACGGCTGTATATTATT GTGCGCGCTCTTACTACGGTTACTTTGACTATTGGG GCCAGGGAACCCTGGTCACCGTCTCCTCA
23	1203, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQYYTVVPFTFGQGTKLEIK
24	1203, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGTACTACACTGTTGTTCCGTT CACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA
25	1205, light chain VL	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQSVPHYPFTFGQGTKLEIK
26	1205, light chain VL	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
			CTTATTACTGTCAACAGTCTGTTCCGCACTACCCGTT CACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA
27	1204, heavy chain VH	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYYMGW VRQAPGKGLEWVSGIGSYYGYTGYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARAYYDYNYYYAYF DYWGQGTLVTVSS
28	1204, heavy chain VH	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTTCTTCTTACTACATGGGTTG GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGG GTCTCAGGTATTGGTTCTTACTACGGTTACACAGGTT ATGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCC GTGACAATTCCAAGAACACGCTGTATCTGCAAATGA ACAGCCTGCGTGCCGAGGACACGGCTGTATATTATT GTGCGCGCGCTTACTACGACTACAACTACTACTACG CTTACTTTGACTATTGGGGCCAGGGAACCCTGGTCA CCGTCTCCTCA
29	1214 (VH)	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWV RQAPGKGLEWVSSIGSGGGYTGYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARVGHPFDYWGQGT LVTVSS
30	1214 (VH)	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTAGCAGCTATGCCATGAGCT GGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG GGTCTCATCTATTGGTTCTGGTGGTGGTTACACAGG TTATGCAGACTCCGTGAAGGGCCGGTTCACCATCTC CCGTGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGCGTGCCGAGGACACGGCTGTATATTA TTGTGCGCGCGTTGGTCATCCGTTTGACTATTGGGG CCAGGGAACCCTGGTCACCGTCTCCTCA
31	1215 (VL)	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQDAYPHTFGQGTKLEIK
32	1215 (VL)	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGGACGCTTACCCGCACACTTT TGGCCAGGGGACCAAGCTGGAGATCAAA
33	1618 (VH)	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSYGSMYW VRQAPGKGLEWVSSISSGSGSTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARSSYYGSYYSIDY

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
			NSKNTLYLQMNSLRAEDTAVYYCARSGYYGSYYGIDY WGQGTLVTVSS
34	1618 (VH)	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTTCTTACGGTTCTATGTACTG GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGG GTCTCATCTATTTCTTCTGGTTCTGGTTCTACATACTA TGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCC GTGACAATTCCAAGAACACGCTGTATCTGCAAATGA ACAGCCTGCGTGCCGAGGACACGGCTGTATATTATT GTGCGCGCTCTTCTTACTACGGTTCTTACTACTCTAT TGACTATTGGGGCCAGGGAACCCTGGTCACCGTCTC CTCA
35	1619 (VL)	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQYYDNLPTFGQGTKLEIK
36	1619 (VL)	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGTACTACGACAACCTGCCCAC TTTTGGCCAGGGGACCAAGCTGGAGATCAAA
37	1620 (VH)	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFSGYYMYW VRQAPGKGLEWVSGISSSGSYTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARSVGPYFDYWGQ GTLVTVSS
38	1620 (VH)	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTTCTGGTTACTACATGTACTG GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGG GTCTCAGGTATTTCTTCTTCTGGTTCTTACACATACT ATGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCC GTGACAATTCCAAGAACACGCTGTATCTGCAAATGA ACAGCCTGCGTGCCGAGGACACGGCTGTATATTATT GTGCGCGCTCTGTTGGTCCGTACTTTGACTATTGGG GCCAGGGAACCCTGGTCACCGTCTCCTCA
39	1621 (VL)	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQGVGPYTFGQGTKLEIK
40	1621 (VL)	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT

SEQ ID NO.	CHAIN NO.	TYPE	SEQUENCE
			ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGGGTGTTGGTCCGTACACTTT TGGCCAGGGGACCAAGCTGGAGATCAAA
41	1626 (VH)	aa	EVQLLESGGGLVQPGGSLRLSCAASGFTFGGYSMYW VRQAPGKGLEWVSSIGGYYYSTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARSYYGSIDYWGQG TLVTVSS
42	1626 (VH)	nt	GAGGTGCAGCTGTTGGAGAGCGGGGGAGGCTTGGT ACAGCCTGGGGGGTCCCTGCGCCTCTCCTGTGCAG CCAGCGGATTCACCTTTGGTGGTTACTCTATGTACT GGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG GGTCTCATCTATTGGTGGTTACTACTACTCTACATAC TATGCAGACTCCGTGAAGGGCCGGTTCACCATCTCC CGTGACAATTCCAAGAACACGCTGTATCTGCAAATG AACAGCCTGCGTGCCGAGGACACGGCTGTATATTAT TGTGCGCGCTCTTACTACGGTTCTATTGACTATTGG GGCCAGGGAACCCTGGTCACCGTCTCCTCA
43	1627 (VL)	aa	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQ KPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQGTGYGPLTFGQGTKLEIK
44	1627 (VL)	nt	GACATCCAGATGACCCAGTCTCCATCCTCCCTGAGC GCATCTGTAGGAGACCGCGTCACCATCACTTGCCGG GCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATC AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCT ATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAC GTTTCAGTGGCAGTGGAAGCGGGACAGATTTCACTC TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAA CTTATTACTGTCAACAGGGTACTGGTTACGGTCCGC TCACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA

Table B - 5T4 antibody sequences - CDR sequences (including claimed and reference sequences)

[0205]

Clone name (mAb)	VH	VL	H1	H2	H3	L1	L2	L3
1206/1207	1206	1207	GFTFSGSS (SEQ ID NO: 45)	IYYSGSGT (SEQ ID NO: 47)	ARYGRNVHPYNLDY (SEQ ID NO: 50)	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQGYYYLPT (SEQ ID NO: 56)
1208/1135	1208	1135	GFTFSSYA (SEQ ID NO: 46)	ISGSGGST (SEQ ID NO: 48)	ARSPYYYGANWIDY (SEQ ID NO: 51)	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQSYSTPYT (SEQ ID NO: 57)
1210/1211	1210	1211	GFTFSSYA (SEQ ID NO: 46)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQTYGYLHT (SEQ ID NO: 58)
1212/1213	1212	1213	GFTFSSYA (SEQ ID NO: 46)	ISSYGGYT (SEQ ID NO: 49)	ARYHSGVLDY (SEQ ID NO: 53)	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQYYYHYLLT (SEQ ID NO: 59)
2992/2993	2992	2993	GFDFESYA (SEQ ID NO: 144)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSIRSA (SEQ ID NO:145)	AAS (SEQ ID NO:55)	QQTYGYLHT (SEQ ID NO:58)
2994/2995	2994	2995	GFDFDSYA (SEQ ID NO:146)	ISGRGGST (SEQ ID NO: 147)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSIRSA (SEQ ID NO:145)	AAS (SEQ ID NO:55)	QQTYGYLHT (SEQ ID NO:58)
2996/2997	2996	2997	GFDFDSYA (SEQ ID NO:146)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSIRQA (SEQ ID NO:148)	AAS (SEQ ID NO: 55)	QQTYGYLHT (SEQ ID NO: 58)
2998/2999	2998	2999	GFDFDSYA (SEQ ID NO: 146)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSISQA (SEQ ID NO: 149)	AAS (SEQ ID NO: 55)	QQTYGYLHT (SEQ ID NO: 58)
3000/3001	3000	3001	GFDFSSYA (SEQ ID	ISGSGGST (SEQ ID NO:	ARYYGGYYSAWMDY	QSIRQA (SEQ ID NO:	AAD (SEQ ID NO:	QQTYGYLHT (SEQ ID

Clone name (mAb)	VH	VL	H1	H2	H3	L1	L2	L3
			NO: 150)	48	(SEQ ID NO: 52)	148)	151)	NO: 58)
3002/3003	3002	3003	GFTFDSYA (SEQ ID NO: 152)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSIRSA (SEQ ID NO: 145)	AAS (SEQ ID NO: 55)	QQTYGYLHT (SEQ ID NO: 58)
3004/3005	3004	3005	GFDFDSYA (SEQ ID NO: 146)	ISGSGGST (SEQ ID NO: 48	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSISSA (SEQ ID NO: 153)	AAS (SEQ ID NO: 55)	QQTYGYLHT (SEQ ID NO: 58)
3006/3007	3006	3007	GFDFESYA (SEQ ID NO: 144)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSIHQA (SEQ ID NO: 154)	GAS (SEQ ID NO: 155)	QQTYGYLHT (SEQ ID NO: 58)
3008/3009	3008	3009	GFDFDSYA (SEQ ID NO: 146)	ISGSGGST (SEQ ID NO: 48)	ARYYGGYYSAWMDY (SEQ ID NO: 52)	QSIHQA (SEQ ID NO: 154)	AAS (SEQ ID NO:55)	QQTYGYLHT (SEQ ID NO: 58)

Table C- CD137 antibodies - CDR sequences (including claimed and reference sequences)

[0206]

Table C(1) - VH

Antibody	CDRH1	CDRH2	CDRH3
1200/1201	GFTFSSYA (SEQ ID NO: 46)	ISGGGGGT (SEQ ID NO: 65)	ARDVAYFDY (SEQ ID NO: 72)
1202/1203	GFTFYGSS (SEQ ID NO: 60)	IYYGSSGT (SEQ ID NO: 66)	ARSYYGYFDY (SEQ ID NO: 73)
1204/1205	GFTFSSYY (SEQ ID NO: 61)	IGSYYGYT (SEQ ID NO: 67)	ARAYYDYNYYYAYFDY (SEQ ID NO: 74)
1214/1215	GFTFSSYA (SEQ ID NO: 46)	IGSGGGYT (SEQ ID NO: 68)	ARVGHPFDY (SEQ ID NO: 75)
1618/1619	GFTFSYGS (SEQ ID NO: 62)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
1620/1621	GFTFSGYY (SEQ ID	ISSSGSYT (SEQ ID	ARSVGPYFDY (SEQ ID NO:

Antibody	CDRH1	CDRH2	CDRH3
	NO: 63)	NO: 70)	77)
1626/1627	GFTFGGYS (SEQ ID NO: 64)	IGGYYYST (SEQ ID NO: 71)	ARSYYGSIDY (SEQ ID NO: 78)
3012/3013	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3014/3015	GFTFSYGS (SEQ ID NO: 62)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3016/3017	GFTFSYGS (SEQ ID NO: 62)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3018/3019	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3020/3021	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3022/3023	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3024/3025	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3026/3027	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3028/3029	GFDFSYGS (SEQ ID NO: 157)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3030/3031	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3032/3033	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3034/3035	GFTFSYGS (SEQ ID NO: 62)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)
3036/3037	GFTFDYGS (SEQ ID NO: 156)	ISSGSGST (SEQ ID NO: 69)	ARSSYYGSYYSIDY (SEQ ID NO: 76)

Table C(2) - VL

Antibody	CDRL1	CDRL2	CDRL3
1200/1201	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQYYIPHT (SEQ ID NO: 79)
1202/1203	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQYYTVVPFT (SEQ ID NO: 80)
1204/1205	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQSVPHYPFT (SEQ ID NO: 81)
1214/1215	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQDAYPHT (SEQ ID NO: 82)
1618/1619	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQYYDNLPT (SEQ ID NO: 83)
1620/1621	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQGVGPYT (SEQ ID NO: 84)

Antibody	CDRL1	CDRL2	CDRL3
1626/1627	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQGTGYGPLT (SEQ ID NO: 85)
3012/3013	QSISQY (SEQ ID NO: 158)	GAS (SEQ ID NO: 155)	QQYYDNLPT (SEQ ID NO: 83)
3014/3015	QSIRQY (SEQ ID NO: 159)	SAD (SEQ ID NO: 160)	QQYYDNLPT (SEQ ID NO: 83)
3016/3017	QSIRQY (SEQ ID NO: 159)	GAS (SEQ ID NO: 155)	QQYYDNLPT (SEQ ID NO: 83)
3018/3019	QSISQY (SEQ ID NO: 158)	SAE (SEQ ID NO: 161)	QQYYDNLPT (SEQ ID NO: 83)
3020/3021	QSIRSY (SEQ ID NO: 162)	SAS (SEQ ID NO: 163)	QQYYDNLPT (SEQ ID NO: 83)
3022/3023	QSIRQY (SEQ ID NO: 159)	GAS (SEQ ID NO: 155)	QQYYDNLPT (SEQ ID NO: 83)
3024/3025	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQYYDNLPT (SEQ ID NO: 83)
3026/3027	QSIRSY (SEQ ID NO: 162)	GAD (SEQ ID NO: 165)	QQYYDNLPT (SEQ ID NO: 83)
3028/3029	QSIRQY (SEQ ID NO: 159)	GAE (SEQ ID NO: 166)	QQYYDNLPT (SEQ ID NO: 83)
3030/3031	QSISSY (SEQ ID NO: 54)	GAE (SEQ ID NO: 166)	QQYYDNLPT (SEQ ID NO: 83)
3032/3033	QSISSY (SEQ ID NO: 54)	AAS (SEQ ID NO: 55)	QQYYDNLPT (SEQ ID NO: 83)
3034/3035	QSISSY (SEQ ID NO: 54)	GAS (SEQ ID NO: 155)	QQYYDNLPT (SEQ ID NO: 83)
3036/3037	QSIRSY (SEQ ID NO: 162)	GAS (SEQ ID NO: 155)	QQYYDNLPT (SEQ ID NO: 83)

Mutated IgG1 antibody sequence

[0207] *IgG1 LALA-sequence*

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAA
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 86)

Linker sequences

[0208]

SGGGGSGGGGS (SEQ ID NO: 87)

SGGGGSGGGGSAP (SEQ ID NO: 88)

NFSQP (SEQ ID NO: 89), KRTVA (SEQ ID NO: 90)

GGGSGGGG (SEQ ID NO: 91)

GGGGSGGGGS (SEQ ID NO: 92)

GGGGSGGGGSGGGGS (SEQ ID NO: 93)

(SG)m, where m = 1 to 7.

IgG constant region sequences

[0209]

IgG1 heavy chain constant region sequence:
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 94)

IgG1 light chain constant region sequence:
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 95)

Table D - Lead optimised VH and VL amino acid sequences for CD137 and 5T4

[0210]

Table D(1) - 5T4-specific VH sequences (optimised sequences from "1210"; SEQ ID NO:9)

2992	EVQLLESGGGLVQPGGSLRLSCAASGFDFESYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 96)**
2994	EVQLLESGGGLVQPGGSLRLSCAASGFDFDSYAMSWVRQAPGKGL EWVSAISGRGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 98)**

2996	EVQLLESGGGLVQPGGSLRLSCAASGFDFDSYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 102)**
2998	EVQLLESGGGLVQPGGSLRLSCAASGFDFDSYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 102)**
3000	EVQLLESGGGLVQPGGSLRLSCAASGFDFSSYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 104)**
3002	EVQLLESGGGLVQPGGSLRLSCAASGFTFDSYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 106)**
3004	EVQLLESGGGLVQPGGSLRLSCAASGFDFDSYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 108)**
3006	EVQLLESGGGLVQPGGSLRLSCAASGFDFESYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 110)**
3008	EVQLLESGGGLVQPGGSLRLSCAASGFDFDSYAMSWVRQAPGKGL EWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARYYGGYYSAWMDYWGQGTLVTVSS **(SEQ ID NO: 112)**

Table D(2) - CD137-specific VH sequences (optimised sequences from "1618"; SEQ ID NO:33)

3012	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 114)**
3014	EVQLLESGGGLVQPGGSLRLSCAASGFTFSYGSMYWVRQAPGKGL EWVSSISSGSGSTHYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 116)**
3016	EVQLLESGGGLVQPGGSLRLSCAASGFTFSYGSMYWVRQAPGKGL EWVSSISSGSGSTHYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 118)**
3018	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 120)**

3020	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 122)**
3022	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 124)**
3024	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 126)**
3026	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTHYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 128)**
3028	EVQLLESGGGLVQPGGSLRLSCAASGFDFSYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 130)**
3030	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTHYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 132)**
3032	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 134)**
3034	EVQLLESGGGLVQPGGSLRLSCAASGFTFSYGSMYWVRQAPGKGL EWVSSISSGSGSTHYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 136)**
3036	EVQLLESGGGLVQPGGSLRLSCAASGFTFDYGSMYWVRQAPGKGL EWVSSISSGSGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCARSSYYGSYYSIDYWGQGTLVTVSS **(SEQ ID NO: 138)**

Table D(3) - 5T4-specific VL sequences (optimised sequences from "1211"; SEQ ID NO:11)

2993	DIQMTQSPSSLSASVGDRVTITCRASQSIRSALNWYQQKPGKAPK LLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 97)**
2995	DIQMTQSPSSLSASVGDRVTITCRASQSIRSALNWYQQKPGKAPK LLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 99)**

2997	DIQMTQSPSSLSASVGDRVTITCRASQSIRQALNWYQQKPGKAPK LLIYAASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 101)**
2999	DIQMTQSPSSLSASVGDRVTITCRASQSISQALNWYQQKPGKAPK LLIYAASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 103)**
3001	DIQMTQSPSSLSASVGDRVTITCRASQSIRQALNWYQQKPGKAPK LLIYAADSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 105)**
3003	DIQMTQSPSSLSASVGDRVTITCRASQSIRSALNWYQQKPGKAPK LLIYAASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 107)**
3005	DIQMTQSPSSLSASVGDRVTITCRASQSISSALNWYQQKPGKAPK LLIYAASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 109)**
3007	DIQMTQSPSSLSASVGDRVTITCRASQSIHQALNWYQQKPGKAPK LLIYGASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 111)**
3009	DIQMTQSPSSLSASVGDRVTITCRASQSIHQALNWYQQKPGKAPK LLIYAASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQT YGYLHTFGQGTKLEIK **(SEQ ID NO: 113)**

Table D(4) - CD137 specific VL sequences (optimised sequences from "1619"; SEQ ID NO: 35)

3013	DIQMTQSPSSLSASVGDRVTITCRASQSISQYLNWYQQKPGKAPK LLIYGASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 115)**
3015	DIQMTQSPSSLSASVGDRVTITCRASQSIRQYLNWYQQKPGKAPK LLIYSADSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 117)**
3017	DIQMTQSPSSLSASVGDRVTITCRASQSIRQYLNWYQQKPGKAPK LLIYGASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 119)**
3019	DIQMTQSPSSLSASVGDRVTITCRASQSISQYLNWYQQKPGKAPK LLIYSAESLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 121)**

3021	DIQMTQSPSSLSASVGDRVTITCRASQSIRSYLNWYQQKPGKAPK LLIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 123)**
3023	DIQMTQSPSSLSASVGDRVTITCRASQSIRQYLNWYQQKPGKAPK LLIYGASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 125)**
3025	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPK LLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 127)**
3027	DIQMTQSPSSLSASVGDRVTITCRASQSIRSYLNWYQQKPGKAPK LLIYGADSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 129)**
3029	DIQMTQSPSSLSASVGDRVTITCRASQSIRQYLNWYQQKPGKAPK LLIYGAESLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 131)**
3031	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPK LLIYGAESLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 133)**
3033	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPK LLIYAASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 135)**
3035	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPK LLIYGASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 137)**
3037	DIQMTQSPSSLSASVGDRVTITCRASQSIRSYLNWYQQKPGKAPK LLIYGASSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQY YDNLPTFGQGTKLEIK **(SEQ ID NO: 139)**

Table D(5) - Connector sequences

Reference	**Amino acid sequence**	**SEQ ID**
m6	GGGGSGGGGS	SEQ ID NO: 92
m15	THTCPPCPEPKSSDK	SEQ ID NO: 140
m16	GGGGS	SEQ ID NO: 141
m17	EAAKEAAKGGGGS	SEQ ID NO: 142
m18	EAAKEAAK	SEQ ID NO: 143

Table D(6) - Additional alterations (modifications)

Reference	Alteration
m2	L234A, L235A Fc mutations
m5	G49C in heavy chain and Q120C in light chain of scFv
m19	P15G, G16N, G17E, S18T in heavy chain of scFv

Table E - Example describing how to translate the Antibody name into a full IgG sequence for bispecific antibodies in Morrison format

[0211]

Antibody name	Composition of construct					
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2)	Connector*	Additional alterations*
1618-1210LO1	1618	1619	2992	2993	m6	m2
* See Table D(5) and D(6) above for details						

[0212] Heavy chain:

[A (underlined); Heavy chain Fc sequence with modification m2; connector m6 (italic); C (bold); linker; D (bold underlined)]

EVQLLESGGGLVQPGGSLRLSCAASGFTFSYGSMYWVRQAPGKGLEWVSSISSGSGST
YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARSSYYGSYYSIDYWGQGTLV
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAP
EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL
PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT
VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*GGGGSGGGGS***EVQLLESGGG**
LVQPGGSLRLSCAASGFDFESYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKG
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCARYYGGYYSAWMDYWGQGTLVTVSSGG
GGSGGGGSGGGGS**DIQMTQSPSSLSASVGDRVTITCRASQSIRSALNWYQQKPGKAP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYGYLHTFGQGTK
LEIK

[SEQ ID NO:167]

[0213] Light chain:

[B (bold underlined); Light chain constant sequence]

DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP
SRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYDNLPTFGQGTKLEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS
TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[SEQ ID NO:168]

EXAMPLES

Example 1 - Selection of 5T4 antibodies from Alligator-GOLD™ library

[0214] Phage display selections against h5T4 were performed using the scFv library ALLIGATOR-GOLD™, a fully human scFv library containing more than 1×10^{10} unique members (Alligator Bioscience AB, Lund, Sweden). Several different selection strategies were employed, including solid phase selection, selection in solution using biotinylated 5T4-Fc, selection with biotinylated 5T4-Fc coupled to streptavidin beads as well as one round of selection against 5T4 expressing B16 cells using a phage stock that previously had been selected against the recombinant h5T4-Fc. Prior to selection, phage stocks were pre-selected against streptavidin, Beriglobin or SLIT2 in order to remove potential binders to streptavidin, the Fc part of the target and binders cross reactive to other leucine rich repeat proteins.

[0215] To identify specific binders from the phage selection, approximately 1250 individual clones were screened in phage format using ELISA coated with 5T4-Fc or non-target protein (Biglycan or Orencia). This was followed by sequence analysis as well as screening as soluble scFv in full-curve ELISA, ELISA performed at 50°C and FACS analysis of selected clones. Based on this, 14 unique candidate scFv were chosen which bound to recombinant 5T4 and to 5T4 expressing cells without showing positive response to non-target molecules or to 5T4 negative cells.

[0216] The selected 14 5T4 scFv clones were converted to full lgG1 for further characterization. A reference anti-5T4 antibody, designated 1628 (selected from a representative prior art disclosure), was used in this study as a positive control.

[0217] Among the 14 clones, four clones were selected for further evaluation in bispecific antibody format. These four clones are described further below, and compared to the reference clone 1628.

Example 2 - Binding to human 5T4 measured by ELISA

Materials and methods

[0218] ELISA was performed using a standard protocol. Plates (#655074, Greiner Bio-One GmbH, Germany) were pre-coated with 0.5 µg/ml 5T4-Fc (obtained from Peter L. Stern, University of Manchester) overnight. 5T4 antibodies were diluted from 6 to 1.5×10^{-3} µg/ml in 1:4 dilutions and added in duplicates of 50 µl to each well. Binding was detected with rabbit anti-h kappa L-chain-HRP (P0129, Dako Denmark) and the ELISA was developed with SuperSignal ELISA PICO Chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL USA) for 2-10 minutes and read in an automated microplate based multi-detection reader (FLUOstar OPTIMA, Netherlands).

Results and conclusions

[0219] The results show that the majority of the 5T4 mAbs bind with similar potency to 5T4 as 1628 (Table 1) with EC50 values in the sub-nM range. However, clone 1208 exhibits a slightly higher EC50 value.

Table 1

Summary of the obtained EC50 in the ELISA of all 5T4 mAb with the confidence intervals and number of experiments			
Clone name	Mean EC50 (nM)	EC50 (nM) 95% Confidence Intervals	n
Reference 1628	0.56	0.3-1.0	8
1206	0.64	0.3-1.4	4
1208	2.24	2.0-2.4	1
1210	0.48	0.2-1.1	4
1212	0.56	0.2-1.2	4

Example 3 - Binding to 5T4 expressed on the cell surface determined by flow cytometry

Materials and methods

[0220] Analysis of 5T4 mAb binding with flow cytometry was performed using 5T4-transfected cell lines and as negative control, mock transfected cells. Three different transfected cell lines used were used for this study; B16, A9 and CHO, transfected either with a 5T4 construct or with an empty vector control construct. Cells were stained with 5T4 antibodies diluted in FACS buffer (PBS, 0.5 % BSA and 0.02% NaN_3). Binding was detected with the secondary antibody anti-IgG (Fc)-PE (109-115-098, Jackson ImmunoResearch Europe, UK) diluted 1:100. Samples were analysed either on a FACSCalibur or a FACSverse (BD Biosciences, Heidelberg, Germany) and mean fluorescence intensity (MFI) determined.

Results and conclusions

[0221] In flow cytometric analysis of 5T4 antibody binding to 5T4-transfected B16 cells, most antibodies show good binding. Large variations in EC50 values between individual experiments were observed. Therefore, results are summarized as mean EC50 in nM as well as mean EC50 normalized to the internal control 1628 (Table 2). An example of dose-response curves for binding of 5T4 mAb to 5T4-transfected B16 cells is shown in Figure 2. In Figure 3, normalized MFI values at a fixed concentration of 2.5 µg/ml antibody is shown. Taken together, the data indicate that most

antibodies bind well to 5T4-transfected B 16 cells, with clone1208 exhibiting weaker binding.

Table 2

Potency of 5T4 antibodies as determined by flow cytometric analysis of 5T4-transfected B16 cells					
	EC50 (nM)		Normalized EC50*		n
Clone	Mean	SD	Mean	SD	
1206	1.8	1.6	3.9	2.7	4
1208	0.7		9.3		1
1210	0.8	0.7	1.6	1.3	4
1212	1.4	2.2	1.1	0.3	4
Reference 1628	1.1	1.4	1.0		4
*EC50 value normalized to 1628					

[0222] In a new attempt to calculate EC50 with flow cytometry a 5T4 mAb dose response experiment was performed using CHO cells stably transfected with human 5T4. A one to four titration series was performed starting from 2.5 nM. The data are summarized in Table 3.

Table 3

Summary of EC50 values, EC50 95% confidence intervals and EC50 normalised to 1628 in flow cytometric analysis of 5T4-transfected CHO cells. Data was normalised and the EC50 values were calculated by nonlinear regression.				
	1206	1208	1210	1628
EC50 nM	0.51	2.07	0.81	0.51
EC50 (95% confidence intervals)	0.2 to 1.2	1.6.to 2.7	0.3 to 2.2	0.14 to 1.8
Normalized to reference 1628	1.0	4.1	1.6	1.0

[0223] Finally, binding potency to 5T4-transfected A9 cells was evaluated in two individual experiments. As in the experiments performed with B16-5T4 cells, the absolute EC50 values determined in individual experiments vary, and data is therefore presented as normalized to the reference 1628 (Table 4). Results indicate that the 5T4 antibodies bind with comparable potency to the reference 1628.

Table 4

Potency of 5T4 antibodies as determined by flow cytometric analysis of 5T4-transfected A9 cells			
Clone	Normalized EC50*		n
	Mean	SD	
1206	2.9	1.9	2
1208	3.9	2.2	2
1210	1.8	0.2	2

Potency of 5T4 antibodies as determined by flow cytometric analysis of 5T4-transfected A9 cells			
Clone	Normalized EC50*		n
1212	0.4	-	1
1628	1.0	-	2
*EC50 value normalized to reference 1628			

[0224] To summarize, the binding potency of four 5T4 antibodies was evaluated by flow cytometry using three different 5T4-transfected cell lines (B16, CHO and A9). The conclusion from these studies is that all antibodies exhibit reasonable binding, with clone 1208 in general exhibiting lower potency than the other clones.

Example 4 - Binding to cynomolgus 5T4

Materials and methods

[0225] The potency of 5T4 antibodies in binding to cynomolgus 5T4 was determined by flow cytometry. CHO cells were stably transfected with *Macaca mulatta* (cynomolgus) 5T4. Cells were stained with 5T4 antibodies diluted in FACS buffer (PBS, 0.5 % BSA and 0.02% NaN_3) using a 1:4 titration starting at 2.5 nM. Binding was detected with the secondary antibody anti-IgG (Fc)-PE (109-115-098, Jackson ImmunoResearch Europe, UK) diluted 1:100. Samples were analysed either on a FACSCalibur or a FACSverse (BD Biosciences, Heidelberg, Germany) and mean fluorescence intensity (MFI) determined. Three experiments were performed with comparable results, although only one experiment included a full dose-response curve whereas the other two experiments included only three antibody concentrations. To compare the EC50 values between human and cynomolgus 5T4, the cy5T4/hu5T4 ratio was calculated from the experiment with the full dose-response.

Results and conclusions

[0226] The three experiments that were performed demonstrate good binding to cynomolgus 5T4 by clones 1206, 1208 and 1210 and weak binding by 1212 and the reference 1628 (Figure 4, Table 5) Clone 1206 had a relatively good potency, but low efficacy. Comparison of the relative EC50 values between cynomolgus and human 5T4 for selected clones shows that clones 1206, 1208 and 1210 have a relatively high affinity for cynomolgus 5T4 whereas 1212 does not.

Table 5

EC50 values for cyno5T4 transfected cells and EC50 95% confidence intervals and the EC50cyno:EC50 human					
Antibody	**1206**	**1208**	**1210**	**1212**	**1628**
EC50 nM	1.53	0.96	0.70	30.7	93.0
EC50 (95% confidence intervals)	1.1 to 2.1	0.5 to 1.8	0.3 to 1.7	21 to 45	37 to 235
Ratio EC50cyno5T4/h5T4	3.0	0.5	0.9	140	182
Data were normalised and the EC50 values were calculated by nonlinear regression					

Example 5 - Affinity determined by surface plasmon resonance

Materials and methods

[0227] Binding kinetics of the 5T4-specific mAbs have been studied using two different SPR-based platforms, the Biacore 3000 (GE Healthcare) and the MASS-1 platform (Sierra Sensors). Briefly, 5T4 was captured at the sensor chip surface either via direct amine coupling (Biacore platform) or using a streptavidin coated chip and biotinylated 5T4 (MASS-1 platform). The different 5T4-specific mAbs were then injected over the chip in increasing concentrations and the association and dissociation rates studied in real time. A 1:1 Langmuir model was used for curve fitting.

Results and conclusions

[0228] A summary of binding rate constants and affinities obtained using the two platforms is presented in Table 6. It should however be taken into consideration that the assay setup used allows for bivalent binding of the mAbs to the antigen. This will give rise to avidity effects that lead to a significant underestimation of the off-rates (kd) and thus also the affinity value (KD). The different 5T4 antibodies show different binding characteristics, with 1208 and the reference 1628 displaying very low off-rates while on-rates vary less between the binders. It is obvious that there are significant variations between the two assays, with an over 10-fold difference for 1206 and 1628. For 1628 this is likely due the difficulty in accurate curve fitting when the off-rate becomes very low (close to no dissociation).

Table 6

Summary of binding kinetics of 5T4-specific mAbs						
	Biacore			MASS-1		
Clone	ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (nM)
1206	1.3E+05	2.8E-04	2.3E-09	1.4E+06	1.3E-04	9.7E-11
1208	-	-	-	2.1E+05	2.1E-06	9.8E-12
1210	5.0E+05	1.0E-04	2.0E-10	5.1E+05	1.9E-04	3.7E-10

Summary of binding kinetics of 5T4-specific mAbs						
	Biacore			MASS-1		
Clone	ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (nM)
1212	4.5E+05	8.1E-04	1.8E-10	-	-	-
1628	6.4E+05	2.6E-08	4.1E-14	1.5E+06	2.1E-06	1.5E-12

Example* 6 - *Domain mapping* of *5T4 antibodies

Materials and methods

[0229] Epitope mapping was performed by investigation of loss of binding by the antibodies using a panel of human/mouse chimeric 5T4 constructs by flow cytometry. This strategy was possible since none of the 5T4 antibodies cross-react with murine 5T4. Two strategies were used for the epitope mapping as illustrated in Figure 5. In one approach, seven human/mouse 5T4 chimeras were constructed based on dividing 5T4 into seven different domains (Figure 5). By replacing each domain with the corresponding mouse sequence seven human/mouse 7 5T4 human/mouse chimeras were generated. The chimeras were generated using the human protein 5T4 sequence NP_006661.1 (reference mRNA sequence NM_006670.4) and the corresponding mouse sequence NP_035757.2 (reference mRNA sequence NM_011627.4). The human/mouse chimeric DNA constructs, as well as human and mouse wild-type 5T4, were cloned into pcDNA3.1 expression vectors. Stably transfected CHO cells were generated and 5T4 expressing cells enriched by MACS sorting, resulting in 60-80% positive cells. In the other approach, cells transfected with a human/mouse 5T4 chimera (Woods et al., 2002, Biochem J 366(1):353-365) was used, in which mouse sequence in amino acid 173-420 replaced the human sequence (Figure 5). As controls human 5T4 and mouse 5T4-transfected cells were used.

[0230] For flow cytometric analysis, cells were stained with different 5T4 antibodies diluted in FACS buffer (PBS, 0.5 % BSA). Binding was detected with the secondary antibody anti-IgG (Fc)-PE (109-115-098, Jackson ImmunoResearch Europe, UK) diluted 1:100. Samples were analysed by FACSverse (BD Biosciences, Heidelberg, Germany) and % positive cells were determined. To compensate for variations in % 5T4 positive cells in the various transfected populations, binding levels were normalized within each chimera by dividing % positive cells for each clone with % positive cells for the clones resulting in the highest % positive cells (% pos $cells_{clonX}$/% positive $cells_{max}$). A normalized value ≤ 0.75 was defined as mAb binding being dependent on the replaced region, whereas a normalized value ≤ 0.25 was defined as complete dependence.

Results and conclusion

[0231] The four 5T4 antibodies were shown to be more or less dependent on at least one of domains E2, E3, E4, E6 or aa 173-420, whereas no clear dependence on E1, E5 or E7 was

observed (Table 7).

[0232] All four antibodies had a distinct binding pattern:

1. 1. Clone 1208; dependent on E2 and E4
2. 2. Clone 1210; dependent on E2, E4 and aa173-420
3. 3. Clone 1206; dependent on E2, E3, E4 and aa173-420
4. 4. Clone 1212 dependent on E6 aa173-420

[0233] The reference antibody 1628 differed from all the exemplary antibodies of the invention, and was completely dependent on E4 and aa173-420.

Table 7

Summary of epitope mapping results summarized as normalized values for one representative experiment.									
Clone	Group	E1	E2	E3	E4	E5	E6	E7	aa173-420
1628		0.96	1.00	1.00	0.06	0.96	0.81	0.91	0.00
1208	1	0.95	0.68	0.96	0.65	1.00	0.94	0.99	1.00
1210	2	0.99	0.02	0.90	0.69	0.89	0.89	0.96	0.00
1206	3	0.96	0.74	0.25	0.52	0.90	0.94	0.94	0.30
1212	4	0.89	0.90	0.93	1.00	0.84	0.03	0.88	-0.01

[0234] The experiment was repeated once for E2, E3 and E6 chimeras and three times for the E4 chimera with high reproducibility. mAbs with a normalized binding value ≤ 0.75 are indicated in bold.

Example 7 - Selection* of CD137 *antibodies from Alligator GOLD® library

[0235] Phage display selections were performed using a human antibody (scFv) library, *Alligator GOLD®* (Alligator Bioscience, Lund, Sweden). Selections towards recombinant CD137 in soluble form, coated onto the surface of beads or tubes, or expressed on the surface of CD137-transfected cells were performed. CTLA4-Fc and an irrelevant His-tagged protein were used as non-targets included in excess in the selections. Priorto each selection round, the phage stocks were pre-selected towards biotinylated beriglobin, CTLA4-Fc, beads or CD137 negative cells to remove unspecific binders.

[0236] To identify specific binders from the phage selection, approximately 4500 individual clones were screened in phage format using ELISA coated with either recombinant target (CD137-Fc) or non-target (CTLA4-Fc) protein, followed by confirmation as soluble scFv for some clones. Clones exhibiting specific binding to CD137 were sequenced and unique clones were produced as IgG for further characterization.

Example 8 - Binding to human* CD137 *measured by ELISA

Material and methods

[0237] Binding of CD137 antibodies to recombinant human CD137 was determined by sandwich ELISA. Briefly, ELISA plates (Greiner # 655074) coated with recombinant human CD137-Fc (R&D # 838-4B) were incubated with serial dilutions of the various CD137 antibodies to be investigated. CD137 antibodies were detected using HRP-conjugated goat-anti-human kappa light chain (AbD Serotec # STAR127P) and developed with SuperSignal ELISA Pico Chemiluminescent substrate (Pierce # 37069). EC50 values of the various antibodies were determined in 2-6 separate experiments.

[0238] Two different reference anti-CD 137 antibodies have been used in this study, as positive controls (designated "1811/1812" and "1813/1814", both of which are available in the art).

Results and conclusion

[0239] The majority of the antibodies exhibit EC50 values in a similar range as those of the reference antibodies, i.e. sub nM or low nM. Data are summarized in Table 8.

Table 8

EC50 values (nM) of Alligator-GOLD-derived CD137 antibodies determined by ELISA for human CD137				
Clone name	**Mean**		**SD**	**n**
1811/1812	0.75		0.137	8
1813/1814	0.33		0.069	5
1200/1201	0.39		0.037	3
1202/1203	0.41		0.050	4
1204/1205	0.34		0.058	6
1214/1215	0.98		0.124	6
1618/1619	0.35		0.018	4
1620/1621	0.38		0.137	2
1626/1627	0.22		0.057	2
n = number of data points.				

***Example 9 - Flow cytometric determination of binding to human and cynomolgus* CD137**

Material and methods

[0240] Binding and EC50 was determined using flow cytometric analysis of CHO cells transfected with human CD137, cynomolgus CD137 or empty vector. The extracellular part of human or cynomolgus CD137 was fused to the transmembrane and intracellular part of human CD40 and cloned into pcDNA3.1. The vector was subsequently stably transfected into CHO cells. Expression of CD137 was confirmed by flow cytometry using CD137 antibody (human CD137-PE, BD Biosciences # 555956) for 30 min at 4°C. CD137-transfected and empty vector-transfected cells were incubated with CD137 antibodies for at least 1h at 4°C to saturate the binding. In order to minimize antibody internalization, 0.05% sodium azide was used in the incubation buffer and all work was performed on ice. The CD137 antibodies were detected using PE-conjugated anti-hIgG antibody (109-115-098, Jackson Immunoresearch laboratories), incubated for 30 min at 4°C. Directly after staining the cells were fixed with a paraformaldehyde solution (10x concentrate BD CellFIX, BD biosciences # 340181). Cells were analyzed by flow cytometry using FACSVerse (BD Biosciences). The median fluorescence intensity (MFI) for each sample was determined and the dose response data was analysed using Graph Pad Prism.

[0241] MFI data was normalized for each antibody, where 0% is defined as the lowest value and 100% is the highest value in the dose titration for each antibody. EC50 and 95% confidence interval were calculated with Graph Pad Prism based on data from the two experiments (non-linear regression (curve fit), constraints set to 0 and 100).

Results and conclusion

[0242] Binding to CHO-huCD137, CHO-cyCD137 and CHO-pcDNA was confirmed in two separate experiments (Figure 6). All CD137 antibodies bind relatively well to human CD137 with EC50 comparable with the two reference antibodies 1811/1812 and 1813/1814. The majority of the CD137 antibodies tested bind well to cynomolgus CD137, except for reference antibody 1811/1812 and 1200/1201 (data not shown) which do not bind at all or very weakly, and clone 1620/1621 which binds weakly and does not reach a complete saturation. It should be noted that the maximum MFI obtained on the cynomolgus CD137 cells were 2-3 fold lower than on the human CD137 expressing cells, which indicate differences in receptor density on the cells.

[0243] The EC50 determination is presented as 95% confidence intervals for each CD137 antibody tested in order to include the inter and intra assay variations (Table 9).

Table 9

95% confidence intervals for the EC50 of each CD137 antibody determined as an average from two experiments of normalized data			
Clone name	**Binding to human CD137, EC50 (µg/mL)**	**Binding to cyno CD137, EC50 (µg/mL)**	**Ratio, cyno:human**
1811/1812	1.00-1.99	Nd	Nd
1813/1814	0.21-0.31	0.13-0.24	0.69
1200/1201	0.20 - 0.36	Nd	Nd
1202/1203	0.16-0.27	0.11-0.17	0.67

Clone name	Binding to human CD137, EC50 (µg/mL)	Binding to cyno CD137, EC50 (µg/mL)	Ratio, cyno:human
1204/1205	0.23-0.39	0.11-0.16	0.43
1214/1215	0.89-1.28	0.41 -0.80	0.54
1618/1619	0.11-0.19	0.086-0.15	0.77
1620/1621	0.20 - 0.42	*3-5**	*14**
1626/1627	0.38-0.67	0.16-0.27	0.41
*The estimated 95% confidence interval is likely underestimated Nd: no data due to incomplete binding to target.			

Example 10 - Affinity* of CD137 *antibodies measured by Biacore

Material and methods

[0244] Human CD137 (R&D systems) was immobilized to the Biacore™ sensor chip, CM5, using conventional amine coupling. The tested antibody and control (serially diluted 1/2 10-0.63 nM) were analyzed for binding in HBS-P (GE, #BR-1003-68) at a flow rate of 30 µl/ml. The association was followed for 5 minutes and the dissociation for 15 minutes. Regeneration was performed twice using 10 mM Glycine pH 1.7 for 30 seconds. The kinetic parameters and the affinity constants were calculated using 1:1 Langmuir model.

Results and conclusion

[0245] The affinities of the antibodies were in the nanomolar to sub-nanomolar range (Table 10) measured using bivalent antibodies flowed over CD137 coated on the chip surface.

Table 10

Kinetic parameters measured by surface plasmon resonance			
Sample	**ka (1/Ms)**	**kd (1/s)**	**KD (M)**
1200	ND	ND	ND
1202	6.76E+05	6.60E-04	9.76E-10
1204	2.54E+05	2.80E-04	1.10E-09
1214	4.54E+04	3.17E-05	6.99E-10
1618	1.02E+06	1.10E-04	1.07E-10
1620	3.92E+05	5.19E-04	1.32E-09
1626	2.32E+05	2.94E-04	1.27E-09
1814	1.05E+06	4.45E-04	4.24E-10

ND; not determined

Example 11 - Target specificity of the CD137 antibodies determined by ELISA

Material and methods

[0246] Binding to TNFR superfamily members for which ELISA methods had already been established (CD40 and OX40) was evaluated to detect potential propensity to cross react to non-target proteins. In addition, a BLAST search was performed identifying TNFRSF21 as the most similar sequence (34% sequence identity). Since this sequence similarity is rather low, determination of non-target binding to OX40 and CD40 was considered sufficient.

[0247] ELISA plates (Greiner # 655074) were coated with 50µl/well of recombinant human OX40 (R&D # 1493-CD), CD40-Fc (Ancell # 504-820) or CD137 (R&D # 838-4B) diluted to a final concentration of 0.5 µg/ml in PBS for 1h at 37°C or overnight at 4°C. Plates were washed with PBS+0.05% TWEEN20 (PBST), followed by block with PBST+1% bovine serum albumin (BSA). Antibody samples were prepared as serial 1/10 dilutions from 10 - 0.01 µg/ml in PBST+1% BSA and incubated for 1h in room temperature, followed by detection using a horse radish peroxidase-conjugated anti-human kappa light chain antibody (AbD Serotec # STAR127P) and developed using SuperSignal ELISA Pico Chemiluminescent substrate (Pierce ThermoScientific #37069).

Results and conclusion

[0248] The results from the two experiments were similar. One antibody (1202/1203) exhibited weak binding to OX40 and CD40, whereas none of the remaining antibody showed any detectable binding to either OX40 or CD40. An overview of antibodies analyzed, and results from the two experiments is shown in Table 11. The EC50 for 1202/1203 binding to CD137 in ELISA was determined as 0.41 nM, corresponding to approx. 0.06 µg/ml. The ELISA signal is very low even at 10 µg/ml, and the EC50 for binding to OX40 and CD40 is most likely higher than 10 µg/ml since the dilution curves have not reached a plateau.

[0249] Further, binding to primary PBL from multiple blood donors was tested. The binding to PBL was similar to Reference antibodies. No relevant unspecific binding to non-target proteins was detected.

Table 11

Summary of CD137 antibody unspecific binding to OX40 and CD40		
pAb	Binding to OX40 and CD40	EC50 CD137
1200/1201	No	
1202/1203	Weak; EC50> 40 nM	0.4 nM
1204/1205	No	

Summary of CD137 antibody unspecific binding to OX40 and CD40		
1214/1215	No	
1618/1619	No	
1620/1621	No	
1626/1627	No	

Example 12 - Domain mapping of antibodies binding to CD137

Material and methods

[0250] The ability of each antibody to bind to a panel of human/mouse CD137 chimeras expressed on the surface of transfected cells was analyzed by flow cytometry.

[0251] The chimeras were designed by exchanging domains or modules of the human CD137 with the corresponding mouse domain (Figure 7). Genes of CD137 human/mouse chimeras were synthesized (GenScript) and constructs cloned into pcDNA3.1 vector (Invitrogen) and transiently transfected into FreeStyle 293-F cells (Invitrogen). The transfected cells were incubated with CD137 antibodies and control antibodies, followed by incubation with anti-human IgG-PE (Jackson Immunoresearch) for detection and analyzed with FACS Verse

[0252] (BD Biosciences). Binding to the different chimeric constructs was calculated as relative MFI compared to the binding of the isotype control, followed by normalization to the full-length human CD137 construct to minimize the effect of affinity differences between individual antibodies.

Results and conclusion

[0253] Four binding patterns can be observed as described below. Data is summarized in Table 12.

Pattern A:

[0254] Antibodies 1811/1812 (Reference antibody) and 1618/1619 are dependent on domain 1.

Pattern B:

[0255] Antibodies 1200/1201, 1202/1203 and 1204/1205 are mainly dependent on domain 2. In addition, some loss of binding is also seen for construct 1555, indicating an impact of domain 1 as well.

Pattern C:

[0256] Antibodies 1813/1814 (Reference antibody) and 1620/1621 appear to be mainly dependent on domains 3B-4A. However, loss of binding is seen for all constructs, making this pattern quite similar to pattern D.

Pattern D:

[0257] For antibodies 1214/1215 and 1626/1627, no clear dependence on particular CD137 domains could be demonstrated. Instead, these antibodies exhibited extensive loss of binding for all chimeras. However, for 1214/1215, the results differed between the experiments (see Table 12).

Table 12

Median fluo full-length h rescen uman C ce inten D137 isity (M FI) for antibody sample/ /isotype contro l, normali zed to										
Group	A		B			C		D		
Domain	1		2			3B-4A		Unclear		
Clone	1811 1812	1618 1619	1200 1201	1202 1203	1204 1205	1813 1814	1620 1621	1626 1627	1214 1215	
									Exp 1	Exp 2
1550	0.12	0.11	0.05	0.05	0.07	0.22	0.17	0.10	0.06	0.14
1551	0.41	0.67	0.04	0.05	0.11	0.37	0.33	0.11	0.07	0.15
1552	0.76	1.20	0.05	0.06	0.13	0.19	0.18	0.11	0.32	0.13
1553	1.07	1.24	0.65	0.65	0.85	0.17	0.17	0.14	0.41	0.15
1554	0.82	1.01	0.84	0.51	0.73	0.16	0.17	0.12	0.26	0.15
1555	0.11	0.12	0.24	0.26	0.28	0.26	0.32	0.29	0.30	0.45
1030*	1	1	1	1	1	1	1	1	1	1
*Full-length CD137										

Example 13 - In vitro efficacy of CD137 antibodies

Material and methods

[0258] Agonistic activity of CD137 antibodies was evaluated in a T cell assay based on primary human $CD8^+$ T cells. Briefly, $CD8^+$ T cells were separated from human peripheral blood mononuclear cells by MACS separation (Miltenyi # 130-096-495) according to the manufacturer's protocol. Cells were incubated in 96-well microtiter plates (NuncThermo Scientific #268200), pre-

coated with anti-CD3 antibody (clone OKT3, Affymetrix eBioscience # 16-0037) and titrated concentrations of the CD137 antibody to be tested. Following 72 or 96 hour incubation, culture medium was harvested and IFN-γ levels were determined by ELISA (BD #555142).

[0259] Each clone was analyzed in at least 6 donors and compared to the reference CD137 antibody 1811/1812 and the negative control antibody.

[0260] Due to large intra-donor variations the stimulation index (SI, fold induction by antibody compared to negative control) was determined for each sample and normalized to the stimulation index for the reference antibody 1811/1812.

Results and conclusion

[0261] Several clones with efficacy comparable to the reference 1811/1812 were identified. Data are summarized in Figure 8, which indicates the absolute IFN-γ levels induced by CD137 stimulation. However, all antibodies were not analyzed head-to-head in all donors, and the normalized SI is more relevant for comparison of the efficacy. The antibodies were evaluated in an IgG1 format, and the efficacy was measured using antibodies coated to the surface of the wells, which may influence the efficacy.

Example 14 - Competitive binding of CD137 antibodies (ligand blocking)

Aim and background

[0262] The aim was to determine if the exemplary CD137 antibodies block the CD137 ligand binding.

[0263] In the previous domain mapping experiment, the CD137 antibodies were divided in different groups based on their binding to similar subdomains of the CD137 antigen. If the CD137 antibodies bind to epitopes close to the ligand binding region, binding to the antigen can lead to partial or total blockade of ligand binding. Binding close to the CD137 ligand binding epitope may also affect the ligand binding due to steric hindrance or conformational changes of the CD137 ligand binding epitope. All CD137 antibodies were titrated against a fixed concentration of CD137L for evaluation of ligand blocking properties.

Material and method

[0264] CHO-cells transfected with human CD137 were used for the ligand competition. The extracellular part of human CD137 was fused to the transmembrane and intracellular part of hCD40 and cloned into pcDNA3.1. The vector was subsequently stably transfected into CHO cells. The expression of CD137 was confirmed by staining with commercial antibody targeting CD137.

[0265] The CHO-huCD137 were pre-incubated with CD137 monoclonal antibodies, titrating down from 10:1 down to 0.01:1 molar ratio CD137 mAb (250 µg/ml) to CD137L (hCD137_CD8 Ligand) (Ancell # 503-020), for 1h at +4C before the addition of CD137 ligand at a concentration at EC50. After co-incubation for another 30 min at +4C, cells were washed and bound CD137 ligand was detected with aCD8a-PE (clone 53-6.7) (BD # 553033) and fixed with paraformaldehyde (10x concentrate BD CellFIX, BD biosciences). Analysis was performed with FACSverse and the MFI (Median Fluorescence Intensity) was calculated with FlowJo software

Results and conclusion

[0266] The CD137L blocking experiment was performed in duplicate. It can be concluded that not all CD137 mAbs tested were blocking the CD137 ligand binding (Table 13, Figure 9). CD137 mAbs belonging to group B and C (1204 and 1620), binding to domain 2B-4A, were blocking the CD137L. Antibody 1814 also blocks the CD137L binding. 1618, belonging to group A which bound to domain 1, did not block CD137 ligand.

Table 13

Maximal CD137 ligand competition of the CD137 antibodies, mean out of two experiments		
Group (domain mapping)	**CD137 mAb**	**CD137L, inhib.**
A	1618	2%
C	1814	67%

Example 15 - Competitive binding* of CD137 *antibodies measured by ELISA

Aim and background

[0267] By competing the exemplary CD137 antibodies with each another, it is possible to determine antibodies binding to similar epitopes based on their blocking pattern. The competition ELISA is performed by co-incubating biotinylated CD137 antibodies with non-biotinylated CD137 antibodies when binding to coated CD137-Fc. Competition is defined as loss of signal from the biotinylated CD137 antibody. Low competition values could either be due to no competition between the antibodies or binding kinetics of the antibodies. Binding of one antibody could also lead to steric hindrance or conformational changes when binding the antigen which affects the binding of the other CD137 antibody.

Material and methods

[0268] CD137 antibodies were biotinylated (EZ-link NHS-LC-Biotin, ThermoFisher) and intact

binding properties to CD137-Fc were verified with ELISA by comparing EC50 between biotinylated and non-biotinylated anti-CD137 mAbs. Non-biotinylated anti-CD137 (anti-CD137-bio) was pre-incubated with CD137-Fc at concentrations 30 times higher than the determined EC50 for 0.5 h. Without washing, anti-CD137-bio was added and co-incubated for another 1h. The binding of anti-CD137-bio was detected with Streptavidin-HRP (Pierce). Competition was calculated as the relative number by dividing the binding measured to other antibodies relative to its maximum competition (competing with itself). The relative values obtained were normalized against the maximum blocking capacity (Table 4).

Table 14

Summary of CD137 antibody competition ELISA from two experiments. Values are presented as % competition with CD137-bio.								
Group comp ELISA	**Pattern X**		**Pattern Y**					
	1812	1618	1202	1204	1814	1620	1626	1214
1812-bio	100	100	7	5	5	4	0	4
1814-bio	15	21	41	74	94	61	57	99
1202-bio	18	19	58	76	63	50	63	92
1214-bio	12	6	81	92	78	80	77	99
1618-bio	84	88	11	3	6	10	16	9
1620-bio	4	7	49	93	100	82	79	100
1626-bio	37	24	100	100	96	97	100	99
1204-bio	23	28	71	88	72	66	66	97

Result and conclusion

[0269] When normalizing the relative competition values for each antibody a competition pattern could be identified (Table 14). The antibodies 1812 and 1618 displayed a unique pattern in the competition ELISA (Pattern X). The other CD137 antibodies that were analyzed had a similar blocking pattern (Pattern Y). Differences in binding kinetics between those antibodies, may explain some of the minor variations in the binding patterns among these antibodies, although it cannot be excluded that the small variations within group Y reflects actual differences in the binding epitope.

Example 16 - Crosslinking dependency* of *CD137 mAbs

Material and methods

[0270] The crosslinking dependency of CD137 antibodies was evaluated in a T cell assay based on primary human $CD8^+$ T cells. Briefly, cells were incubated in 96-well microtiter plates (NuncThermo

Scientific #268200) pre-coated with anti-CD3 antibody (clone OKT3, Affymetrix eBioscience # 16-0037). Titrated concentrations of CD137 antibodies in the presence and absence of crosslinking antibody, goat-anti human Fc F(ab')2 (Jackson Immuno #109-006-098) at 1:3 molar ratio were added to the plated. Following 72, culture medium was harvested and IFN-γ levels were determined by ELISA (BD #555142).

Results and conclusion

[0271] The results are summarised in Figure 10 showing the CD137 activation in the presence of crosslinking antibody and Figure 11 showing the activation in the absence of crosslinking antibody. From the results obtained it can be concluded that CD137 mAb clone 1618 is crosslinking dependent and the reference, a CD137 specific IgG4 antibody (REF Ab), is crosslinking independent, when it comes to CD137 mediated activation of CD137-expressing immune cells.

Example 17 - Production of 5T4-CD137 bispecific antibodies

Materials and methods

[0272] Thirty 5T4-CD137 bsAb, based on four 5T4 and eight CD137 antibodies were cloned as bsAb with one of the binding moieties cloned as a scFv and fused to the C-terminus of heavy chain of the IgG (i.e. in the Morrison format). The majority of the bsAb were clones with the 5T4 binder as scFv and the CD137 agonist as IgG, but in some constructs, a CD137 scFv was fused to the heavy chain of a 5T4 IgG (Table 15). In addition, four isotype control constructs, where either the 5T4 or the CD137 binder had been replaced with an isotype control antibody were included. bsAb were produced by transient transfection of Freestyle293 cells (Thermo Fischer) and purified by Protein A chromatography.

[0273] The bsAb designation was as follows:

- First number indicates antibody clone name
- Second number indicates scFv clone name

[0274] Thus, the designation "1200-1206" refers to the 5T4 binder 1206 (i.e. comprising the variable domain heavy and light chain sequences of antibody 1206/1207) in scFv format fused to the C-terminus of the Fc of the CD137 agonist antibody 1200 (i.e. comprising the variable domain heavy and light chain sequences of antibody 1200/1201).

Table 15

List of all 5T4-CD137 bsAb that were cloned and produced for further evaluation					
	Protein name	**mAb target**	**mAb (clone name)**	**scFv target**	**scFv (clone name)**
1	1200-1206	CD137	1200	5T4	1206
2	1200-1208	CD137	1200	5T4	1208
3	1200-1210	CD137	1200	5T4	1210
4	1200-1212	CD137	1200	5T4	1212
5	1202-1206	CD137	1202	5T4	1206
6	1202-1208	CD137	1202	5T4	1208
7	1202-1210	CD137	1202	5T4	1210
8	1202-1212	CD137	1202	5T4	1212
9	1204-1206	CD137	1204	5T4	1206
10	1204-1208	CD137	1204	5T4	1208
11	1204-1210	CD137	1204	5T4	1210
12	1204-1212	CD137	1204	5T4	1212
13	1210-1202	5T4	1210	CD137	1202
14	1210-1204	5T4	1210	CD137	1204
15	1210-1214*	5T4	1210	CD137	1214
16	1212-1202	5T4	1212	CD137	1202
17	1212-1204	5T4	1212	CD137	1204
18	1212-1214	5T4	1212	CD137	1214
19	1206-1202	5T4	1206	CD137	1202
20	1206-1204	5T4	1206	CD137	1204
21	1208-1202	5T4	1208	CD137	1202
22	1208-1204	5T4	1208	CD137	1204
23	1214-1208	CD137	1214	5T4	1208
24	1618-1208	CD137	1618	5T4	1208
25	1620-1208	CD137	1620	5T4	1208
26	1626-1208	CD137	1626	5T4	1208
27	1214-1210	CD137	1214	5T4	1210
28	1618-1210	CD137	1618	5T4	1210
29	1620-1210	CD137	1620	5T4	1210
30	1626-1210	CD137	1626	5T4	1210
	1862-1210	Isotype control	1862	5T4	1210
	1862-1212	Isotype control	1862	5T4	1212
	1202-1862	CD137	1202	Isotype	1862

List of all 5T4-CD137 bsAb that were cloned and produced for further evaluation					
	Protein name	**mAb target**	**mAb (clone name)**	**scFv target**	**scFv (clone name)**
				control	
	1204-1862	CD137	1204	Isotype control	1862
*No expression					

Example 18 - Binding to human CD137 and 5T4 by 5T4-CD137 bispecific antibodies measured by ELISA

Materials and methods

[0275] Bispecific binding to both targets, CD137 and 5T4, was evaluated using a standard ELISA protocol. Plates (#655074, Greiner Bio-One GmbH, Germany) were pre-coated with 0.5 µg/ml 5T4-Fc (obtained from Professor Peter Stern, University of Manchester) overnight. CD137-5T4 bsAb were diluted from 8 to 2 $\times 10^{-3}$ µg/ml in 1:4 dilutions and added in duplicates of 50 µl to each well. CD137-bio (Ancell #502-030) was used as detection antibody at 0.5 µg/ml and the binding was detected with Streptavidin-HRP (Pierce #21126). The ELISA was developed with SuperSignal ELISA PICO Chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL USA) during 2-10 minutes and read in an automated microplate based multi-detection reader (FLUOstar OPTIMA, Netherlands).

Results and conclusions

[0276] The majority of the bsAb bound to both targets in dual ELISA with EC50 values at sub- or low nM range. However, some bsAb exhibited considerably higher EC50 values, indicating poor affinity to either or both targets in this antibody - scFv combination. Dose response curves are shown in Figure 12 and EC50 values are summarized in Table 16.

Table 16

EC50 values of dual binding of 5T4-CD137 bispecific antibodies		
Clone	EC50 (nM)	95% Confidence Intervals (nM)
1200-1206	2.17	1.3 - 3.7
1202-1206	0.61	0.3 - 1.2
1204-1206	0.54	0.3 - 0.9
1206-1202	0.28	0.2 - 0.4
1206-1204	0.66	0.4 - 1.1
1200-1208	16.82	7.9 - 35.8

EC50 values of dual binding of 5T4-CD137 bispecific antibodies		
Clone	EC50 (nM)	95% Confidence Intervals (nM)
1202-1208	2.25	1.1 - 4.7
1204-1208	2.34	1.2 - 4.6
1214-1208	1.40	0.5 - 4.1
1618-1208	1.47	0.4 - 5.2
1620-1208	12.70	5.7 - 28.4
1626-1208	1.91	0.6 - 5.9
1208-1202	1.32	0.7 - 2.5
1208-1204	1.21	0.7 - 2.1
1200-1210	3.73	2.6 - 5.3
1202-1210	1.20	0.6 - 2.4
1204-1210	0.73	0.4 - 1.4
1214-1210	0.19	0.1 - 0.5
1618-1210	0.20	0.1 - 0.6
1620-1210	1.74	0.7 - 4.1
1626-1210	0.40	0.1 - 1.2
1210-1202	0.28	0.1 - 0.6
1210-1204	0.28	0.1 - 0.5
1200-1212	1.55	1.0 - 2.3
1202-1212	1.67	1.0 - 2.7
1204-1212	1.01	0.6 - 1.6
1212-1202	0.57	0.4 - 0.9
1212-1204	0.27	0.2 - 0.5
1212-1214	0.79	0.5 - 1.3

Example 19 - Affinity of CD137-5T4 bispecific antibodies measured by surface plasmon resonance

Materials and methods

[0277] Binding kinetics of a selection of the CD137-5T4 bsAbs was evaluated using the SPR-based MASS-1 platform (Sierra Sensors). Briefly, CD137 or 5T4 was captured at the sensor chip surface using a streptavidin coated chip and biotinylated antigen. The different CD137-5T4 bsAbs were then injected over the chip in increasing concentrations and the association and dissociation rates studied in real time.

Results and conclusions

[0278] A summary of the obtained binding rate constants and affinities obtained is presented in Table 17. It should be taken into consideration that the assay setup used allows for bivalent binding of the bsAbs to the antigen. This will give rise to avidity effects that lead to a significant underestimation of the off-rates (kd) and thus also the affinity value (KD). This makes comparisons to other compounds troublesome, but the obtained values are valid for comparisons within the dataset.

[0279] The results from the kinetics analysis confirm retained affinity of the CD137-specific mAb part of the bispecific molecule, while the scFv part displays reduced 5T4 affinity as compared to the parental mAb. As expected, the conformational changes induced by a flexibility reducing linker in the scFv format has a negative effect on the antigen binding affinity. In the case of 1210 this effect is only minor, while the affinity of 1208 is reduced about 6 times.

Table 17

Summary of binding kinetics of CD137/5T4-specific bsAbs						
bsAb	**Antigen**	**Fit model**	**ka (1/Ms)**	**kd (1/s)**	**KD (M)**	**parental mAb KD (M)**
1618-1208	CD137	1:1 Langmuir	9.40E+05	1.09E-04	1.16E-10	1.56E-10
1618-1208	5T4	1:1 Langmuir	7.65E+04	4.62E-05	6.01E-10	9.63E-11
1618-1210	CD137	1:1 Langmuir	1.26E+06	1.62E-04	1.29E-10	1.56E-10
1618-1210	5T4	1:1 Langmuir	4.86E+05	4.55E-04	9.37E-10	4.37E-10

Example 20 - Functional activity* of *5T4-CD137 bispecific antibodies on human CD8+ T cells cultured in 5T4-Fc coated plates

Materials and methods

[0280] The functional activity of 5T4-CD137 bsAb was evaluated in a CD8 T cell assay, where cells were cultured in microtiter plates coated with 5T4-Fc and CD3 antibody. Peripheral blood mononuclear cells (MNC) were isolated by density gradient centrifugation using Ficoll-Paque (p 1.077g/ml) (GE Healthcare #17-1440-02) from leucocyte concentrates obtained from healthy donors (Clinical Immunology and Transfusion Medicine, Labmedicin Region Skåne, Lund Sweden). $CD8^+$T cells were enriched by negative selection using the $CD8^+$ T cell isolation kit (Miltenyi 130-096-495). Plates were coated overnight at 4°C with 3 µg/ml aCD3, clone OKT3 (Affymetrix eBioscience #16-0037-85), washed and coated with 5 µg/ml 5T4-Fc for 2 h at 37 °C. After the 5T4-

Fc coating, plates were washed and blocked for a minimum of 30 minutes with RPMI (Gibco # 61870010) containing 10 % FCS (Heat inactivated, Gibco # 10270-106 lot 41Q9248K) and 10 mM Hepes (Gibco # 15630056).

[0281] CD137-5T4 bsAbs were diluted in RPMI containing 10% FCS and 10 mM Hepes and added to the plates 30 minutes before addition of $CD8^+$ T cells (0.07×10^6 cells/well). Assay plates were incubated for either 68 or 92 h at 37 °C, and culture supernatant harvested. IFN-γ levels in the supernatants were measured by ELISA (BD OptiEIA #555142). Results are shown as fold change compared to CE_1200-1210, which was used as an internal control in all experiments.

Results and conclusions

[0282] Results from the first set of bsAbs used at a fixed concentration of 1µg/ml (Figure 13) show that the majority of the bsAbs based on either of the 5T4 binders 1206, 1208 or 1210 were functional in the T cell assay, whereas those that were based on the 5T4 antibody 1212 were not. Data also suggest that bsAb based on CD137 clone 1202 may have lower efficacy and/or potency compared to bsAb based on CD137 clones 1204 and 1200. The agonistic effect of the 5T4-CD137 bsAb was dependent on cross-linking by 5T4, since no activation was obtained in the absence of 5T4 or using bsAb comprising one isotype control moiety.

[0283] Based on these results, a second set of bsAbs based on five new CD137 clones as IgG and the 5T4 clones 1208 and 1210 as scFv were investigated. 5T4 binders 1212 and 1206 were excluded due to poor functional activity as bsAb, and low Tm value and thus poor stability as scFv, respectively. Functional activity of all bsAb is summarized in Figure 14.

Example 21 - Functional activity of 5T4-CD137 bispecific antibodies on human CD8+ T cells cultured with 5T4-expressing tumor cells

Materials and methods

[0284] CD8 T cells were isolated as described above, and cultured in the presence of 5T4-expressing cells B16 cells. B16 cells transfected with empty vector were used as negative control. CD3 stimulation was performed with aCD3 (OKT-3) coated beads (Dynal M-450 Tosylactivated #14013) according to the manufactures protocol.

[0285] Irradiated B16 tumor cells (6000 cells/well) were added to the 96 well plates and let to attached for 2 h. CD137-5T4 bsAbs were added and incubated for 30 minutes prior to addition of $CD8^+$ T cells (0.1×10^6 cells/well) and aCD3 coated beads (0.5×10^5 beads/well). Plates were cultured for 68 or 92 h and IFN-γ levels in the media measured by ELISA (BD OptiEIA #555142).

Results and conclusions

[0286] Results from the fully cell-based assay show that the majority of the bsAb are functional and that the effect is 5T4-specific, with no activation induced by 5T4 negative B 16 cells or isotype-CD137 bsAb (Figure 15).

Example 22 - Optimization of affinity and biophysical properties of bispecific antibodies: Optimization of 5T4-specific variable domains

Material and methods

[0287] The aim of the optimization was to generate versions of the 5T4-specific 1210/1211 antibody in regards to affinity and biophysical properties. Selections were performed towards 5T4 with lead optimized library 1210LOlib1. In total, 170 unique clones were identified in the initial primary screening with good target signal as well as target/non-target ratio. These clones were further investigated in an extended primary screening with regards to temperature stability. The temperature stability evaluation showed that the majority of the identified unique clones displayed a better stability compared to the wild type 1210/1211 scFv clone. The top 96 clones were further evaluated in a dose-response ELISA and they all showed a similar and acceptable binding behaviour. Sequence analysis from the test-screening and primary screening showed similar trends.

[0288] The top 96 identified clones from the primary and the extended primary screening were further re-cloned as the scFv-part in the Morrison-format, with 1618/1619 as the monoclonal antibody (mAb) part, and were evaluated based on binding, affinity and stability.

[0289] Kinetic measurements were performed using the Octet RED96 platform equipped with Anti-human Fab-CH1 2nd generation sensor tips (ForteBio). Bispecific antibodies were diluted to 1.5 µg/ml in 1x kinetic buffer (ForteBio) and coupled to the biosensors. Human 5T4 (produced in-house) was diluted in 1x Kinetics Buffer to 50 nM, 10 nM and 2 nM. Binding kinetics were studied in 1x Kinetics buffer where association was allowed for 300 sec followed by dissociation for 600 sec. Sensor tips were regenerated using 10 mM glycine, pH 2.2. Data generated were referenced by subtracting a parallel buffer blank, the baseline was aligned with the y-axis, inter-step correlation by alignment against dissociation was performed and the data were smoothed by a Savitzky-Golay filter in the data analysis software (v.9.0.0.14). The processed data were fitted using a 1:1 Langmuir binding model with X^2 as a measurement of fitting accuracy.

Results and conclusions

[0290] The data are summarised in Table 18. Overall, the lead optimized variants behaved very similarly in the EC50 evaluation in ELISA. The affinity evaluation showed that the affinity (KD) had been improved between 3 to more than 10 times compared to the wild type 1618-1210 clone. The EC50 evaluation on cells also showed similar behaviour of the different optimized variants and all showed an improved performance compared to the wild type 1618-1210 clone. In regards to

stability, the top performing clones show less than 10% aggregation after protein A purification and have a Tm higher than the Tm of the Fc (>70 °C) as measured by HPLC and DSF respectively.

Table 18

Summary of affinity measurements of optimized 5T4-specific variable domains									
Antibody name	Composition of construct (amino acid sequences)						Affinity measurements Octet (5T4)		
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2)	Connector*	Additional alteration*	KD (M)	kon(1/Ms)	kdis(1/s)
1618-1210LO1	1618	1619	2992	2993	m6	m2	1,47E-10	2,23E+05	3,29E-05
1618-1210LO2	1618	1619	2994	2995	m6	m2	2,70E-10	1,67E+05	4,50E-05
1618-1210LO3	1618	1619	2996	2997	m6	m2	3,49E-10	2,12E+05	7,40E-05
1618-1210LO4	1618	1619	2998	2999	m6	m2	3,82E-10	2,02E+05	7,73E-05
1618-1210LO5	1618	1619	3000	3001	m6	m2	4,67E-10	1,64E+05	7,65E-05
1618-1210LO6	1618	1619	3002	3003	m6	m2	4,69E-10	2,27E+05	1,06E-04
1618-1210LO7	1618	1619	3004	3005	m6	m2	4,95E-10	1,68E+05	8,29E-05
1618-1210LO8	1618	1619	3006	3007	m6	m2	4,98E-10	1,77E+05	8,83E-05
1618-1210LO9	1618	1619	3008	3009	m6	m2	5,34E-10	2,35E+05	1,25E-04
* see Table D(5) and D(6) for details									

Example 23 - Optimization of affinity and biophysical properties of bispecific antibodies: Optimization of CD137-specific variable domains

Material and methods

[0291] The aim of the optimization was to generate versions of the CD137-specific 1618/1619 antibody in regards to affinity and biophysical properties. Selections were performed towards CD137 with lead optimized library 1618LOlib1. In total, 153 unique clones were identified in the initial primary screening with good target signal as well as target/non-target ratio. These clones were further investigated in an extended primary screening with regards to temperature stability. The temperature stability evaluation allowed for identification of the best performing unique clones in

regards to temperature stability compared to the wild type 1618/1619 scFv clone. The top 50 clones were further evaluated in a dose-response ELISA and they all showed a similar and acceptable binding behaviour. Sequence analysis from the test-screening and primary screening showed similar trends.

[0292] The top 50 identified clones from the primary and the extended primary screening were further re-cloned as the scFv-part in the Morrison-format, with 1210/1211 as the monoclonal antibody (mAb) part, and were evaluated based on binding, affinity and stability.

[0293] Kinetic measurements were performed using the Octet RED96 platform equipped with Anti-human Fab-CH1 2nd generation sensor tips (ForteBio). Bispecific antibodies were diluted to 1.5 µg/ml in 1x kinetic buffer (ForteBio) and coupled to the biosensors. Human CD137-Fc (R&D Systems, #838-4B) was diluted in 1x Kinetics Buffer to 50 nM, 10 nM and 2 nM. Binding kinetics were studied in 1x Kinetics buffer where association was allowed for 300 sec followed by dissociation for 600 sec. Sensor tips were regenerated using 10 mM glycine, pH 2.2. Data generated were referenced by subtracting a parallel buffer blank, the baseline was aligned with the y-axis, inter-step correlation by alignment against dissociation was performed and the data were smoothed by a Savitzky-Golay filter in the data analysis software (v.9.0.0.14). The processed data were fitted using a 1:1 Langmuir binding model with X^2 as a measurement of fitting accuracy.

Results and conclusions

[0294] The data are summarised in Table 19. Overall, the lead optimized variants behaved very similarly in the EC50 evaluation in ELISA. The affinity evaluation showed that the affinity (KD) were comparable to the wild type 1210-1618 clone. The EC50 evaluation on cells also showed similar behaviour of the different optimized variants. In regards to stability, the top performing clones show less than 6% aggregation after protein A purification and have a Tm between 54°C - 59 °C as measured by HPLC and DSF respectively.

Table 19

Summary of affinity measurements of optimized CD137-specific variable domains									
Antibody name	Composition of construct (amino acid sequences)						Affinity measurements Octet (CD137)		
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2)	Connector*	Additional alteration*	KD (M)	kon(1/Ms)	kdis(1/s)
1210-1618LO1	1210	1211	3012	3013	m6	m2	1,58E-09	2,18E+05	3,44E-04
1210-1618LO2	1210	1211	3014	3015	m6	m2	1,64E-09	2,81E+05	4,61E-04
1210-1618LO3	1210	1211	3016	3017	m6	m2	1,90E-09	3,45E+05	6,54E-04
1210-1618LO4	1210	1211	3018	3019	m6	m2	2,38E-09	2,89E+05	6,87E-04

Summary of affinity measurements of optimized CD137-specific variable domains									
Antibody name	Composition of construct (amino acid sequences)						Affinity measurements Octet (CD137)		
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2)	Connector*	Additional alteration*	KD (M)	kon(1/Ms)	kdis(1/s)
1210-1618LO5	1210	1211	3020	3021	m6	m2	2,56E-09	2,62E+05	6,72E-04
1210-1618LO6	1210	1211	3022	3023	m6	m2	2,57E-09	2,99E+05	7,68E-04
1210-1618LO7	1210	1211	3024	3025	m6	m2	2,86E-09	2,85E+05	8,14E-04
1210-1618LO8	1210	1211	3026	3027	m6	m2	2,98E-09	2,19E+05	6,53E-04
1210-1618LO9	1210	1211	3028	3029	m6	m2	3,24E-09	3,32E+05	1,08E-03
1210-1618L010	1210	1211	3030	3031	m6	m2	3,38E-09	2,80E+05	9,48E-04
1210-1618LO11	1210	1211	3032	3033	m6	m2	3,66E-09	2,89E+05	1,06E-03
1210-1618LO12	1210	1211	3034	3035	m6	m2	2,38E-09	3,83E+05	9,11E-04
1210-1618LO13	1210	1211	3036	3037	m6	m2	2,82E-09	3,52E+05	9,95E-04
* see Table D(5) and D(6) for details									

Example 24 - Optimization of affinity and biophysical properties of bispecific antibodies: Dual ELISA analysis of optimized bispecific antibodies

Material and methods

[0295] Optimized bispecific antibodies with improved biophysical properties were obtained using different strategies including combining lead optimized binding domains and the use of additional mutations and connectors.

[0296] The bispecific antibody in this example is an IgG-scFv bispecific antibody. The CD137 binding domain is an intact IgG and the 5T4 binding domain is an scFv attached to the C-terminus of a heavy chain of the IgG. The bispecific antibodies comprise for example the following components: (1) Two heavy chains each comprising, in order from the N-terminus to the C terminus: [a VH sequence; A in Table 20] - [an H chain constant region of IgG1 subtype with no

mutations unless stated by an mX suffix in Table 20] - [an m6, m15, m16 or m17 connector] - [a scFv, wherein the variable chains (heavy or light) are ordered from the N-terminus to the C terminus so that chain C in Table 20 is followed by a linker and then followed by Chain D in Table 20]; and (2) Two light chains each comprising, in order from the N-terminus to the C terminus: [a VL sequence; B in Table 20] - [an L chain constant region].

[0297] The scFv for some of the bispecific antibodies in this example carry recombinant N-glycosylation sites placed either in the

[0298] Optimized bispecific antibody encoding genes were designed in house and synthesized at GeneArt (Thermo Fisher, Life Technologies) or generated by standard cloning methods into expression vectors. Bispecific antibodies were produced by transient transfection of Expi293™ (Thermo Fischer Scientific) and purified by Protein A chromatography. Bispecific binding to both targets, CD137 and 5T4, was evaluated using a standard ELISA protocol. Plates (#655074, Greiner Bio-One GmbH, Germany) were pre-coated with 0.5 µg/ml 5T4-Fc (obtained from Professor Peter Stern, University of Manchester) overnight. CD137-5T4 bsAb were diluted from 8 to 2 $\times 10^{-3}$ µg/ml in 1:4 dilutions and added in duplicates of 50 µl to each well. CD137-bio (Ancell #502-030) was used as detection antibody at 0.5 µg/ml and the binding was detected with Streptavidin-HRP (Pierce #21126). The ELISA was developed with SuperSignal ELISA PICO Chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL USA) during 2-10 minutes and read in an automated microplate based multi-detection reader (FLUOstar OPTIMA, Netherlands).

Results and conclusions

[0299] The data are summarised in Table 20. The optimized bispecific antibodies, consisting of lead optimized CD137 binding domains and/or lead optimized 5T4 binding domains and/or stabilised bispecific antibodies using novel connectors and/or additional stabilising strategies including reversed heavy and light chain order or N-glycosylation sites, display dual binding for both targets, CD137 and 5T4. Binding domains with improved binding such as for example 1210LO1 and 1210LO2 provide improved dual binding as observed as lower EC50 values compared to bispecific antibodies comprising non-optimized 1210 binding domains.

Table 20

Summary of Dual ELISA measurements of optimized CD137/5T4-specific bsAbs							
Antibody name	Composition of construct (amino acid sequence)						EC50 Dual ELISA
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2)	Connector*	Additional alteration*	
1618LO1-1210LO1	3012	3013	2992	2993	m6	m2	0,5
1618LO1-1210LO2	3012	3013	2994	2995	m6	m2	0,5
1618LO3-1210LO1	3016	3017	2992	2993	m6	m2	0,5
1618LO3-1210LO2	3016	3017	2994	2995	m6	m2	0,4

Summary of Dual ELISA measurements of optimized CD137/5T4-specific bsAbs							
Antibody name	Composition of construct (amino acid sequence)						EC50 Dual ELISA
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2)	Connector*	Additional alteration*	
1618LO3-1210	3016	3017	1210	1211	m6	m2	0,9
1618LO11-1210LO1	3032	3033	2992	2993	m6	m2	0,5
1618LO11-1210LO2	3032	3033	2994	2995	m6	m2	0,6
1618LO11-1210	3032	3033	1210	1211	m6	m2	1,1
1210-1618.m2.m15	1210	1211	1618	1619	m15	m2	0,4
1210-1618.m2.m16	1210	1211	1618	1619	m16	m2	0,4
1210-1618.m2.m7.m15	1210	1211	1619	1618	m15	m2	0,7
1210-1618.m2.m7.m17	1210	1211	1619	1618	m17	m2	0,6
1210-1618.m2.m7.m18	1210	1211	1619	1618	m18	m2	0,4
1618-1210.m2.m15	1618	1619	1210	1211	m15	m2	0,5
1618-1210.m2.m16	1618	1619	1210	1211	m16	m2	0,5
1618-1210.m2.m17	1618	1619	1210	1211	m17	m2	0,5
1618-1210.m2.m7.m15	1618	1619	1211	1210	m15	m2	5,3
1618-1210.m2.m7.m16	1618	1619	1211	1210	m16	m2	3,7
1618-1210.m2.m7.m17	1618	1619	1211	1210	m17	m2	1,2
1618-1210.m2.m7.m18	1618	1619	1211	1210	m18	m2	2,6
1618-1210.m2.m6.m19	1618	1619	1210	1211	m6	m2, m19	0,4
1618-1210.m2.m6.m20	1618	1619	1210	1211	m6	m2, m20	0,4
* see Table D(5) and D(6) for details							

Example 25 - Binding of lead optimised 5T4 clones to cells expressing 5T4, measured by flow cytometry

Materials and methods

[0300] Analysis of 5T4 mAb binding with flow cytometry was performed using human and *Macaca mulatta* (cynomolgus) 5T4-transfected CHO-K cell lines and as negative control, mock transfected cells. Cells were stained with 5T4 lead optimised clones (scFv in bsAb format) diluted in FACS buffer (PBS, 0.5 % BSA and 0.02% NaN_3). Binding was detected with the secondary antibody anti-IgG (Fc)-PE (109-115-098, Jackson ImmunoResearch Europe, UK) diluted 1:100. Samples were run on a FACSverse (BD Biosciences, Heidelberg, Germany) and mean fluorescence intensity (MFI) was determined using the FlowJo software.

[0301] ELISA was performed using a standard protocol. Plates (#655074, Greiner Bio-One GmbH, Germany) were pre-coated with 0.5 µg/ml 5T4-Fc (produced in-house) overnight. 5T4 antibodies were diluted in PBST+1% BSA and 50 µl was added to each well. Binding was detected with anti-human kappa light chain antibody (AbD Serotec # STAR127P) and the ELISA was developed with SuperSignal ELISA PICO Chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL USA) for 2-10 minutes and read in an automated microplate based multi-detection reader (FLUOstar OPTIMA, Netherlands).

Results and conclusions

[0302] Binding curves for CHOh5T4 and CHOcyno5T4 cells can be seen in Figure 16 (A and B). All the lead optimized variants have similar binding potency towards both human and cyno 5T4 expressing cells as well as towards human 5T4 measured with ELISA compared to the original antibody. The lead optimized variants have an improved affinity for both human and cyno 5T4 as measured with both ELISA and FACS.

Example 26 - Binding of lead optimised CD137 clones to cells expressing CD137, measured by flow cytometry

Material and methods

[0303] Analysis of CD137 mAb binding with flow cytometry was performed using human and cyno CD137-transfected CHO-K cell lines and as negative control, mock transfected cells. Cells were stained with CD137 lead optimised clones (as scFv in bsAb format) diluted in FACS buffer (PBS, 0.5 % BSA and 0.02% NaN_3). Binding was detected with the secondary antibody anti-IgG (Fc)-PE (109-115-098, Jackson ImmunoResearch Europe, UK) diluted 1:100. Samples were run on a FACSverse (BD Biosciences, Heidelberg, Germany) and mean fluorescence intensity (MFI) was determined using the FlowJo software.

[0304] ELISA plates (Greiner # 655074) were coated with 50µl/well of recombinant CD137 (R&D # 838-4B) diluted to a final concentration of 0.5 µg/ml in PBS for 1h at 37°C or overnight at 4°C. Plates were washed with PBS+0.05% TWEEN20 (PBST), followed by block with PBST+1% bovine serum albumin (BSA). Antibody samples were diluted in PBST+1% BSA and incubated for 1h in room temperature, followed by detection using a horse radish peroxidase-conjugated anti-human kappa light chain antibody (AbD Serotec # STAR127P) and developed using SuperSignal ELISA Pico Chemiluminescent substrate (Pierce ThermoScientific #37069).

Results and conclusions

[0305] Binding curves of the bispecific antibodies to CHOhCD137 and CHOcynoCD137 cells can be seen in Figure 17 (A and B).

[0306] The EC50 values are comparable to the wild type 1210-1618 (1618 as scFv) clone for both human and cyno CD137.

Example 27 - In vitro activity of lead optimised 5T4-CD137 (1618-1210) bispecific antibodies in an IFNγ release assay using human CD8+ T cells on 5T4 coated plates

Material and methods

[0307] The functional activity of the 5T4-CD137 bsAb was evaluated in a CD8+ T cell assay, where cells were cultured in microtiter plates coated with 5T4-Fc and CD3 antibody. Peripheral blood mononuclear cells (MNC) were isolated by density gradient centrifugation using Ficoll-Paque (p 1.077g/ml) (GE Healthcare #17-1440-02) from leucocyte concentrates obtained from healthy donors (Clinical Immunology and Transfusion Medicine, Labmedicin Region Skåne, Lund Sweden). CD8+T cells were enriched by negative selection using the CD8+ T cell isolation kit (Miltenyi 130-096-495). Plates were coated overnight at 4°C with 3 µg/ml aCD3, clone OKT3 (Affymetrix eBioscience #16-0037-85), washed and coated with 5 µg/ml 5T4-Fc for 2 h at 37°C. After the 5T4-Fc coating, plates were washed and blocked for a minimum of 30 minutes with RPMI (Gibco # 61870010) containing 10% FCS (Heat inactivated, Gibco # 10270-106 lot 41Q9248K) and 10 mM Hepes (Gibco # 15630056). 1618-1210 bsAb was diluted in RPMI containing 10% FCS and 10 mM Hepes and added to the plates 30 minutes before addition of CD8+ T cells (0.07 x 10^6 cells/well). Assay plates were incubated for 68 h at 37°C, and culture supernatant harvested. IFN-γ levels in the supernatants were measured by ELISA (BD OptiEIA #555142).

Results and conclusions

[0308] The potency of the 1618-1210 bsAb was determined to EC50 0.6-0.9 nM using human CD8+ T cells cultured in 5T4-Fc coated plates and was based on two experiments and a total of six

donors. The data were normalised and the EC50 was determined using a three-parameter sigmoidal dose-response model (Figure 18).

[0309] Bispecific antibodies variants with optimized variable domains of 1618-1619 and 1210-1211 were generated as outlined in Table D, Table E, Table 18, Table 19 and Table 20. The generated bsAbs variants with optimized variable sequences were functional in the CD8+ T cell assay with crosslinked 5T4-Fc as seen in Figure 19. To correlate the results from the different assay plates the calculated IFNγ levels were normalised to a plate reference.

[0310] Bispecific antibodies were also generated with different linkers as outlined in the Table D, Table E and Table 20, and were evaluated in the CD8 T cell assay. As shown in Figure 20 the generated bsAbs could induce a CD137 activation only in the presence of the tumour antigen. As in previous Figure 19, the obtained IFNγ values were normalised to a positive control in the plate.

Example 28 - In vitro activity of lead optimised 5T4-CD137 (1618-1210) bispecific antibodies in an IFNγ release assay using human CD8+ T cells cultured with 5T4-expressing tumor cells (B16-5T4)

Material and methods

[0311] CD8+ T cells were isolated as described above, and cultured in the presence of tumour associated antigen (TAA) 5T4-expressing cells B16 cells. B16 cells transfected with empty vector were used as negative control. CD3 stimulation was performed with aCD3 (OKT3) coated beads (Dynal M-450 Tosylactivated #14013) according to the manufacturer's protocol.

[0312] Irradiated B16 tumour cells (6000 cells/well) were added to the 96 well plates and left to attach for 2 hours. CD137-5T4 bsAbs were added and incubated for 30 minutes prior to addition of CD8+ T cells (0.1×10^6 cells/well) and aCD3 coated beads (0.5×10^5 beads/well). Plates were cultured for 68 hours and IFN-γ levels in the media were measured by ELISA (BD OptiEIA #555142).

Results and conclusions

[0313] The bispecific antibody 1618-1210 induced IFNγ production in CD8+ T cells in a dose dependent manner when cultured on cells expressing 5T4 (TAA), but not when cultured on cells that do not express 5T4. The results further confirm that CD137-TAA antibodies stimulate T cells only in the presence of tumour antigens. The potency of 1618-1210 bsAb was determined to EC50 0.2-0.7 nM in the CD8+ T cell assay performed with B16-5T4 expressing tumour cells. The EC50 was based on the normalised data from two donors and determined by using a three-parameter sigmoidal dose-response model (Figure 21).

Example 29 - In vitro activity of lead optimised 5T4-CD137 (1618-1210) bispecific antibodies

in an IFNγ release assay using human PBMCs cultured in 5T4-Fc coated plates

Material and methods

[0314] The functional activity of 5T4-CD137 bsAb was evaluated in a PBMC assay, where cells were cultured in microtiter plates coated with 5T4-Fc antibody. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque (p 1.077g/ml) (GE Healthcare #17-1440-02) from leucocyte concentrates obtained from healthy donors (Clinical Immunology and Transfusion Medicine, Labmedicin Region Skåne, Lund Sweden). CD8+T cells were enriched by negative selection using the CD8+ T cell isolation kit (Miltenyi 130-096-495). Plates were coated with 5 µg/ml 5T4-Fc for 2 h at 37°C. After the 5T4-Fc coating, plates were washed and blocked for a minimum of 30 minutes with RPMI (Gibco # 61870010) containing 10 % FCS (Heat inactivated, Gibco # 10270-106 lot 41Q9248K) and 10 mM Hepes (Gibco # 15630056).

[0315] 1618-1210 bsAb were diluted in RPMI containing 10% FCS and 10 mM Hepes and added to the plates 30 minutes before addition of CD8+ T cells (0.1x 10^6 cells/well). CD3 stimulation was performed with 1 µg/ml soluble aCD3. Assay plates were incubated for 68 hours at 37°C, and culture supernatant harvested. IFN-γ levels in the supernatants were measured by ELISA (BD OptiEIA #555142).

Results and conclusions

[0316] The results shown in Figure 22 demonstrate that the bispecific antibody 1618-1210.m2 induced a TAA (5T4)-dependent CD137 mediated activation of PBMCs. No activation of PBMCs was detected without 5T4 present in the assay.

Example 30 - In vitro activity of lead optimised 5T4-CD137 bispecific antibodies in an IFNγ release assay using human CD8+ T cells cultured with CD32 (FcγRII)-expressing L cells

Material and methods

[0317] CD8 T cells were isolated as described above, and cultured in the presence of CD32-expressing L cells. CD3 stimulation was performed with aCD3 (OKT3) coated beads (Dynal M-450 Tosylactivated #14013) according to the manufacturer's protocol.

[0318] Irradiated CD32 L cells (10000 cells/well) were added to the 96 well plates and left to attach for 2 hours. CD137 (1618) mAb with and without the LALA mutation was added and incubated for 30 minutes prior to addition of CD8+ T cells (0.1 ×10^6 cells/well) and aCD3 coated beads (0.5 ×10^5 beads/well). Plates were cultured for 68 hours and IFN-γ levels in the media were measured by

ELISA (BD OptiEIA #555142).

Results and conclusions

[0319] Results from the co-culture assay of CD32-expressing cells with CD8+ T cells, shown in Figure 23, demonstrate that CD137 activation is only induced by 1618 containing the wt **IgG1** and not by the Fc silenced 1618 IgG1 containing the LALA mutation, further supporting the conclusion that activation of T cells via CD137 with antibodies such as 1618/1619 requires cross linking.

Example 31— Binding* of *TAA-CD137 bispecific antibodies measured by dual-binding ELISA

Material and methods

[0320] Bispecific antibodies against three tumor associated antigens (TAA), EpCAM, HER2 and EGFR were generated. A scFv (1204/1205) binding to CD137 was fused to the C-terminal end of three different IgG antibodies with the sequences corresponding to the binding domains of Edrecolomab, Cetuximab and Herceptin. bsAbs were produced by transient transfection of Expi293™ (Thermo Fischer Scientific) and purified by Protein A chromatography.

[0321] Bispecific binding to both targets, CD137 and TAA, was evaluated using a standard ELISA protocol. Plates (#655074, Greiner Bio-One GmbH, Germany) were pre-coated with 0.5 µg/ml TAA (hEGFR-His, SinoBiological#10001-H08H, hEpCAM-Fc, SinoBiological #10694-H02H, HER2-His, SinoBiological#10004-H08H and 5T4-Fc) overnight. TAA-bsAb were diluted from 20 µg/ml in 1:4 dilutions and added in duplicates of 50 µl to each well. CD137-bio (Ancell #502-030) was used as detection antibody at 0.5 µg/ml and the binding was detected with Streptavidin-HRP (Pierce #21126). The ELISA was developed with SuperSignal ELISA PICO Chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL USA) during 2-10 minutes and read in an automated microplate based multi-detection reader (FLUOstar OPTIMA, Netherlands).

Results and conclusions

[0322] The generated TAA-CD137 bsAbs bound to both targets in the dual ELISA with EC50 values in the low nM range (Table 21, Figure 24).

Table 21

Summary of the generated TAA-CD137 bsAbs		
mAb	**scFv**	**bsAb name**
EpCam (2414)	CD137 (1204)	2414-1204
EGFR (2424)	CD137 (1204)	2424-1204
Her2 (2078)	CD137 (1204)	2078-1204

Reference Example 32 - In vitro activity of TAA-CD137 bispecific antibodies in an IFNγ release assay using human CD8+ T cells cultured in TAA coated plates

Material and methods

[0323] The functional activity of the three TAA-CD137 bsAbs binding to EpCAM, EGFR and Her2 was evaluated in a CD8+ T cell assay, where cells were cultured in microtiter plates coated with CD3 antibody and either EGFR, EpCam or Her2. As negative controls, parallel wells were coated with only CD3 antibody. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (p 1.077g/ml) (GE Healthcare #17-1440-02) from leucocyte concentrates obtained from healthy donors (Clinical Immunology and Transfusion Medicine, Labmedicin Region Skåne, Lund Sweden). CD8+T cells were enriched by negative selection using the CD8+ T cell isolation kit (Miltenyi 130-096-495). Plates were coated overnight at 4°C with 3 µg/ml aCD3, clone OKT3 (Affymetrix eBioscience #16-0037-85), washed and coated with 5 µg/ml TAA for 2 h at 37°C. After the TAA coating, plates were washed and blocked for a minimum of 30 minutes with RPMI (Gibco # 61870010) containing 10% FCS (Heat inactivated, Gibco #10270-106 lot 41Q9248K) and 10 mM Hepes (Gibco #15630056).

[0324] TAA-CD137 bsAbs were diluted in RPMI containing 10% FCS and 10 mM Hepes and added to the plates 30 minutes before addition of CD8+ T cells (0.07 x 10^6 cells/well). Assay plates were incubated for 68 hours at 37°C, and culture supernatant harvested. IFN-γ levels in the supernatants were measured by ELISA (BD OptiEIA #555142).

Results and conclusions

[0325] The functionality of the EpCAM-1204 (Figure 25(A)), EGFR-1204 (Figure 25(B)) and Her2-CD137 (Figure 25(C)) bsAbs was analysed and it was concluded that all of the generated bsAbs induced TAA mediated CD137 activation in the presence of TAA and not in the absence of TAA (wells coated with only CD3 antibody). This strongly indicates that the TAA-dependent CD137-mediated immune cell activation generated by CD137-TAA antibodies is a general phenomenon applicable to all types of cell surface expressed TAA.

Example 33 - In vivo anti-tumor effect of bispecific antibody 1618-1210 in a CT26-5T4 colon cancer model

Summary

[0326] The anti-tumor effect of 1618-1210 (an exemplary antibody targeting CD137 and 5T4) was

investigated using transgenic mice for human CD137 and subcutaneous syngeneic tumor model of CT26 colon carcinoma expressing human 5T4.

[0327] The bispecific antibody 1618-1210 demonstrated tumor volume inhibition compared to monoclonal antibody 1618 targeting CD137.

Material and methods

[0328] Human 4-1BB knock-in mouse model was developed by Prof. Lieping Chen and heterozygote F1 females were used in the experiments. The heterozygotes were generated by breeding male homozygotes for human CD137 in C57 background together with BalbC females. All experiments were done by approval of Malmö/Lund ethical committee.

[0329] CT26 colon cancer cells were obtained from ATCC and transfected with human 5T4. The CT26-5T4 cell line growing in log phase was injected subcutaneously (0.5×10^6 cells in 100µL on day 0 (D0)) into the right hind/flank. Intraperitoneal treatments (1.33nM) were done on days 7, 10, and 13.

[0330] The tumor was measured in width, length and height with a calliper, of which the tumor volume was calculated (w/2 × l/2 × h/2 × pi × (4/3)). The animals were terminated before the tumor volume reached $2cm^3$, at wounding, or affected health of the mice.

[0331] The data were analysed for tumor volume inhibition by the bispecific antibody compared to the monoclonal antibody using GraphPad Prism and Excel.

Results and conclusions

[0332] Anti-tumor efficacy was demonstrated using treatment with bispecific antibody 1618-1210 compared to treatment with monoclonal antibody 1618 at days 8-22 in the form of tumor growth inhibition. The percentage of tumor volume inhibition ranged from 0-68% when treated with 1618-1210 (see Table 22).

[0333] In conclusion, the anti-tumor effect of 1618-1210 was investigated using transgenic mice for human CD137 and a subcutaneous tumor model of CT26 colon carcinoma transfected with human 5T4. The bispecific antibody 1618-1210 demonstrated tumor volume inhibition compared to monoclonal CD137 mAb 1618.

Table 22

Tumor inhibition	
Day after tumor inoculation	**Tumor growth inhibition (tumor volume) by bispecific Ab compared to monospecific Ab**
D0	0%
D6	5%

Tumor inhibition	
Day after tumor inoculation	**Tumor growth inhibition (tumor volume) by bispecific Ab compared to monospecific Ab**
D8	17%
D10	43 %
D13	68 %
D15	65 %
D17	50 %
D20	45 %
D22	45 %

Example 34 - Bispecific 5T4-CD137 antibodies localize to the tumor area

Material and methods

[0334] Female SCID-Beige mice (7-8 w) from Taconic (Denmark) were used in the experiments. All experiments were done by approval of the Malmö/Lund ethical committee.

Twin tumor studies (B16 and CT26 tumors)

[0335] B16.F10 wt (B16) melanoma was obtained from ATCC and cultivated according to recommendations by ATCC. B16-5T4 was obtained from Professor Peter Stern and cultivated in the same medium, supplemented with 1.2 mg/mL G418. CT26 and CT26-5T4 cells were cultivated in RPMI, 10% FCS, NaPy and HEPES. CT-5T4 medium was supplemented with 1.2 mg/mL G418.

[0336] For B16 and CT26 tumors, twin tumor studies were performed and each mouse received one 5T4 negative and one positive tumor at each side of the flank. The cell lines, growing in log phase, were injected subcutaneously (1×10^5 cells in 100 µL at day 0). Human PBMCs (10×10^6 in 100 µL), isolated from leukocyte concentrates, were injected intraperitoneally on the same day. Leukocyte concentrates were obtained from Lund University Hospital.

[0337] Intraperitoneal antibody treatments (100 µg) were done on days 6 and 13 for B16 tumors and days 6, 13 and 20 for CT26 tumors.

Single tumor studies (SKOV-3 tumors)

[0338] For SKOV-3 tumors, each mouse received a single tumor in the right flank. The cell line, growing in log phase, was injected subcutaneously (10×10^6 cells in 100 µL on day 0). Human

PBMCs (10×10^6 in 100 µL), isolated from leukocyte concentrates, were injected intraperitoneally once the average tumor volume reached above 100 mm^3. Intraperitoneal antibody treatments (100 µg) were done starting at 6 days after the PBMC transfer, on days 55, 62 and 67.

FACS analysis

[0339] Mice were sacrificed 24 h after the final treatment and tumors were collected. Tumors were enzymatically digested using Liberase TL (Roche #05401020001) and DNase I (Roche #10104159001). Digested tumor material was passed through a 70 µm cell strainer (Fisher Scientific #22363548) and the resulting single cell suspension was stained for FACS analysis.

[0340] Unspecific antibody binding was blocked using mouse IgG (Jackson ImmunoResearch #015-000-003) and Fc block (BD #553141). Dead cells were detected using Fixable Viability Stain 450 (BD #562247) according to manufacturer's instructions. Binding of antibody (human IgG) to the tumor cells was detected using goat-anti-human IgG - PE (Jackson ImmunoResearch #109-115-098). Samples were run on a FACSVerse (BD) and data were analysed using FlowJo software.

Results

Localization of 1618-1210.m2.m5 to 5T4-positive B16 and CT26 tumors in SCID-Beige mice

[0341] Binding of human IgG was clearly detectable on approximately 6% of the cells in B16-5T4 tumors from mice treated with 1618-1210.m2.m5, but not in B16.F10 wt tumors from the same mice. Mice treated with 1618.m2 or 2112.s4.m3 had human IgG bound to <1% of cells irrespective of tumor type (Figure 26).

[0342] A similar observation was made in CT26 tumor-bearing mice. Antibody localization was again observed specifically in 5T4-expressing tumors. Similar to B16 tumors, human IgG was detectable in approximately 8% of the viable tumor cells in mice treated with 1618-1210.m2.m5, but not with 1618.m2 or 2112.s4.m3. Additionally, biotinylated CD137 was also bound specifically by cells from 5T4-expressing tumors, from mice treated with 1618-1210.m2.m5 (Figure 27).

Localization of 1618-1210.m2.m5 to SKOV-3 tumors

[0343] Similar to what was observed for CT26 tumors, binding of biotinylated CD137 was observed in SKOV-3 tumors from mice treated with 1618-1210.m2.m5, but not 1618.m2 or 2112.s4.m3. Additionally, cells that had bound CD137 also expressed 5T4, suggesting that the bispecific antibody targets specifically 5T4-expressing cells and remains intact within the tumor (Figure 28).

Conclusion

[0344] These data show that the bispecific antibody 1618-1210.m2.m5 binds selectively to 5T4-expressing tumors *in vivo.* In contrast, the CD137 monospecific antibodies 1618.m2 and 2112.s4.m3 do not localize to the tumors.

Example 35 - Enhanced Tm of optimized 5T4 and CD137 binders

[0345] Tm measurements were performed on soluble scFv or bispecific antibodies using protein fluorescence with the UNcle platform (Unchained Labs). Onset of aggregation was measured with static light scattering (SLS) with the UNcle platform. Measurements were performed in the temperature range 20 °C - 95 °C with ramping speed of 0.4 °C per min. in PBS and at a protein concentration range of 0.12-1.32 mg/ml. The data analysis was performed with the UNcle Analysis software version 2.0 using default settings.

[0346] As can be seen in Table 23, the optimized sequences exhibit improved Tm1 and Tagg compared to the wildtype sequences (1618 and 1210, respectively).

[0347] In Table 24, the Tm1 and Tagg for exemplary bispecific antibodies (in Morrison format) is displayed. The data shows that the thermostability is increased when the optimized sequences are employed in the bispecific format.

Table 23

scFv antibody name	Sequence ID		Tm1 (°C)	Tagg (°C)
1618	1618	1619	56.9	51.8
1210	1210	1211	72.7	70.5
1210LO1	2992	2993	78.8	75.0
1618LO1	3012	3013	59.5	58.0
1618LO3	3016	3017	58.3	56.3
1618LO11	3032	3033	60.3	58.8
1618LO12	3034	3035	58.8	56.9
1618LO13	3036	3037	60.5	58.1

Table 24

Antibody name	Composition of construct (amino acid sequences)						Tm1 (°C)	Tagg (°C)
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2	Connector*	Additional alterations*		
1618LO1-1210LO1	3012	3013	2992	2993	m6	m2	74.7	74.5
1618LO1-1210LO2	3012	3013	2994	2995	m6	m2	73.2	72.6
1618LO1-1210	3012	3013	1210	1211	m6	m2	71.1	71.1
1618LO3-1210LO1	3016	3017	2992	2993	m6	m2	73.5	73.5

Antibody name	Composition of construct (amino acid sequences)						Tm1 (°C)	Tagg (°C)
	A (VH of B1)	B (VL of B1)	C (VH of B2)	D (VL of B2	Connector*	Additional alterations*		
1618LO3-1210LO2	3016	3017	2994	2995	m6	m2	73.0	72.8
1618LO3-1210	3016	3017	1210	1211	m6	m2	71.7	70.8
1618LO11-1210LO1	3032	3033	2992	2993	m6	m2	74.1	74.4
1618LO11-1210LO2	3032	3033	2994	2995	m6	m2	73.5	73.7
1618LO11-1210	3032	3033	1210	1211	m6	m2	71.3	71.9
* See Tables D(5) and D(6) for details								

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HIDTIL UKENDTE BISPECIFIKKE POLYPEPTIDER MOD CD137

Patentkrav

1. Bispecifikt antistof, der omfatter et første bindingsdomæne betegnet B1, som er i stand til specifikt at binde sig til CD137, og et andet bindingsdomæne betegnet B2, som er i stand til specifikt at binde sig til et tumorcelle-associeret antigen, hvor det tumorcelle-associerede antigen er 5T4;

hvor bindingsdomænet B1 omfatter CDRL1 svarende til SEQ ID NO: 54, CDRL2 svarende til SEQ ID NO: 55 og CDRL3 svarende til SEQ ID NO: 83, og

hvor bindingsdomænet B1 omfatter CDRH1 svarende til SEQ ID NO: 62 eller en variant af CDRH1, som kun har én aminosyremutation i forhold til SEQ ID NO: 62, CDRH2 svarende til SEQ ID NO: 69 og CDRH3 svarende til SEQ ID NO: 76; og

hvor bindingdomænet B2 omfatter CDRL1 svarende til SEQ ID NO: 54 eller en variant af CDRL1, som kun har én aminosyremutation i forhold til SEQ ID NO: 54, CDRL2 svarende til SEQ ID NO: 55 og CDRL3 svarende til SEQ ID NO: 58, og

hvor bindingdomænet B2 omfatter CDRH1 svarende til SEQ ID NO: 46, CDRH2 svarende til SEQ ID NO: 48 og CDRH3 svarende til SEQ ID NO: 52.

2. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor:

(a) bindingsdomænet B1 og/eller bindingsdomænet B2 er et intakt IgG-antistof;

(b) bindingsdomænet B1 og/eller bindingsdomænet B2 er et Fv-fragment; og/eller

(c) bindingsdomænet B1 og/eller bindingsdomænet B2 er et Fab-fragment;

og/eller

hvor det bispecifikke antistof omfatter en human Fc-region eller en variant af denne region, hvor regionen er en IgG1-, IgG2-, IgG3- eller IgG4-region, fortrinsvis en IgG1- eller IgG4-Fc-region; eventuelt

hvor den humane Fc-region udviser ingen eller mindst 10 gange reduceret affinitet over for FcgRI, FcgRII og FcgRIII ved sammenligning med humant vildtype-IgG1 som bestemt ved den koncentration, hvor der opnås halv maksimal binding ved flowcytometrisk analyse af FcyR-udtrykkende celler ved FcyR-ELISA; og/eller

hvor den humane Fc-region er en variant af en human IgG1-Fc-region, der omfatter en mutation i en eller flere af følgende positioner: L234, L235, P239, D265, N297 og/eller P329; eventuelt

hvor alanin er til stede i den/de muterede position(er), såsom de dobbelte mutationer L234A og L235A.

3. Bispecifikt antistof ifølge krav 2, hvor det bispecifikke antistof er valgt fra grupperne bestående af:

(a) bivalente bispecifikke antistoffer, såsom IgG-scFv-bispecifikke antistoffer (for eksempel hvor B1 er et intakt IgG, og B2 er et scFv bundet til B1 i den N-terminale ende af en let kæde og/eller i den C-terminale ende af en let kæde og/eller i den N-terminale ende af en tung kæde og/eller i den C-terminale ende af en tung kæde af IgG'et, eller hvor B2 er et intakt IgG, og B1 er et scFv bundet til B2 i den N-terminale ende af en let kæde og/eller i den C-terminale ende af en let kæde og/eller i den N-terminale ende af en tung kæde og/eller i den C-terminale ende af en tung kæde af IgG'et);

(b) monovalente bispecifikke antistoffer, såsom et bispecifikt "knob-in-hole"-antistof (for eksempel et scFv-KIH, scFv-KIH^r, et BiTE-KIH eller et BiTE- KIH^r;

(c) bispecifikke scFv$_2$-Fc-antistoffer;

(d) bispecifikke BiTE/scFv$_2$-antistoffer;

(e) bispecifikke DVD-Ig-antistoffer eller andre bispecifikke IgG-FAb-, FAb-IgG-antistoffer;

(f) DART$_2$-Fc eller DART-Fc;

(g) bispecifikke DNL-Fab$_3$-antistoffer og

(h) bispecifikke scFv-HSA-scFv-antistoffer.

4. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor bindingsdomænet B1 og bindingsdomænet B2 er direkte fusioneret med hinanden; og/eller hvor bindingsdomænet B1 og bindingsdomænet B2 er forbundet vha. en polypeptidlinker; eventuelt

hvor linkeren er valgt fra gruppen bestående af aminosyresekvensen SGGGGSGGGGS (SEQ ID NO: 87), SGGGGSGGGGSAP (SEQ ID NO: 88), NFSQP (SEQ ID NO: 89), KRTVA (SEQ ID NO: 90), GGGSGGGG (SEQ ID NO: 91), GGGGSGGGGS (SEQ ID NO: 92), GGGGSGGGGSGGGGS (SEQ ID NO: 93), THTCPPCPEPKSSDK (SEQ ID NO: 140), GGGS (SEQ ID NO: 141), EAAKEAAKGGGGS (SEQ ID NO: 142), EAAKEAAK (SEQ ID NO: 143) eller (SG)m, hvor m = 1 til 7.

5. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor det bispecifikke antistof er ude af stand til at inducere antistofafhængig cellulær cytotoksicitet (ADCC), antistofafhængig cellulær fagocytose (ADCP) og/eller komplementafhængig cytotoksicitet (CDC); og/eller

hvor det bispecifikke antistof deraf er i stand til at inducere tumorimmunitet; og/eller

hvor det bispecifikke antistof deraf er i stand til at inducere:

(a) aktivering af cytotoksiske T-celler, dvs. CD8+ T-celler;

(b) aktivering af hjælpe-T-celler, dvs. $CD4^+$ T-celler;

(c) aktivering af dendritceller; og/eller

(d) aktivering af naturlige dræberceller; og/eller

(e) omprogrammering af Tregs til effektor-T-celler.

6. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor bindingsdomænet B1 binder sig til human CD137 med en K_D-værdi på mindre end 10×10^{-9}M, for eksempel mindre end 4×10^{-9}M eller mindre end $1{,}2 \times 10^{-9}$M, og/eller hvor bindingsdomænet B2 binder sig til det tumorcelle-associerede antigen med en K_D-værdi på mindre end 10×10^{-9}M, for eksempel mindre end 4×10^{-9}M eller mindre end $1{,}2 \times 10^{-9}$M; hvor K_D-værdien er målt ved overfladeplasmonresonansanalyse.

7. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor bindingsdomænet B1 omfatter den variable letkæderegion SEQ ID NO: 35 og den variable tungkæderegion SEQ ID NO: 33 eller en variant, som udviser mere end 60 %, eller mere end 70 %, f. eks. 75 eller 80 %, fortrinsvis mere end 85 %, f. eks. mere end 90 eller 95 % aminosyreidentitet med SEQ ID NO: 35 og/eller SEQ ID NO: 33.

8. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor bindingsdomænet B2 omfatter den variable letkæderegion SEQ ID NO: 11 og den variable tungkæderegion SEQ ID NO: 9 eller en variant, som udviser mere end 60 %, eller mere end 70 %, f. eks. 75 eller 80 %, fortrinsvis mere end 85 %, f. eks. mere end 90 eller 95 % aminosyreidentitet med SEQ ID NO: 11 og/eller SEQ ID NO: 9.

9. Bispecifikt antistof ifølge et hvilket som helst af de foregående krav, hvor:

(a) B1 omfatter den variable letkæderegion SEQ ID NO: 35 og/eller den variable tungkæderegion SEQ ID NO: 33, og B2 omfatter den variable letkæderegion SEQ ID NO: 11 og/eller den variable tungkæderegion SEQ ID NO: 9; eller

(b) B1 og/eller B2 omfatter varianter af de variable letkæderegioner og/eller de variable tungkæderegioner, som udviser mindstt 90 % sekvensidentitet dermed.

10. Isoleret nukleinsyremolekyle, der koder for et bispecifikt antistof ifølge et hvilket som helst af de foregående krav; eventuelt hvor molekylet er et cDNA-molekyle.

11. Vektor, der omfatter et nukleinsyremolekyle ifølge krav 10; eventuelt hvor vektoren er en ekspressionsvektor.

12. Rekombinant værtscelle, der omfatter et nukleinsyremolekyle ifølge krav 10 eller en vektor ifølge krav 11; eventuelt hvor værtscellen er en bakteriecelle eller en pattedyrscelle eller en human celle.

13. Fremgangsmåde til fremstilling af bispecifikt antistof ifølge et hvilket som helst af kravene 1 til 9, hvilken fremgangsmåde omfatter dyrkning af en værtscelle som defineret i krav 12 under forhold, der tillader ekspression af det bispecifikke antistof.

14. Farmaceutisk sammensætning, der omfatter en effektiv mængde bispecifikt antistof ifølge et hvilket som helst af kravene 1 til 9 og en farmaceutisk acceptabel fortynder, bærer eller excipiens; eventuelt tilpasset til parenteral indgivelse eller tilpasset til intravenøs indgivelse.

15. Bispecifikt antistof ifølge et hvilket som helst af kravene 1 til 9 til anvendelse i medicin.

16. Bispecifikt antistof ifølge et hvilket som helst af kravene 1 til 9 til anvendelse ved behandling eller forebyggelse af en neoplastisk lidelse hos et individ; eventuelt hvor den neoplastiske lidelse er forbundet med dannelsen af solide tumorer i individets krop; eventuelt

hvor den solide tumor er valgt fra gruppen bestående af prostatacancer, brystcancer, lungecancer, kolorektal cancer, melanomer, blærecancer, cancer i hjernen/centralnervesystemet, cervixcancer, øsofaguscancer, mavecancer, hoved-

halscancer, nyrecancer, levercancer, lymfomer, ovariecancer, pancreascancer, sarkomer og renalcellekarcinom.

DRAWINGS

Figure 1

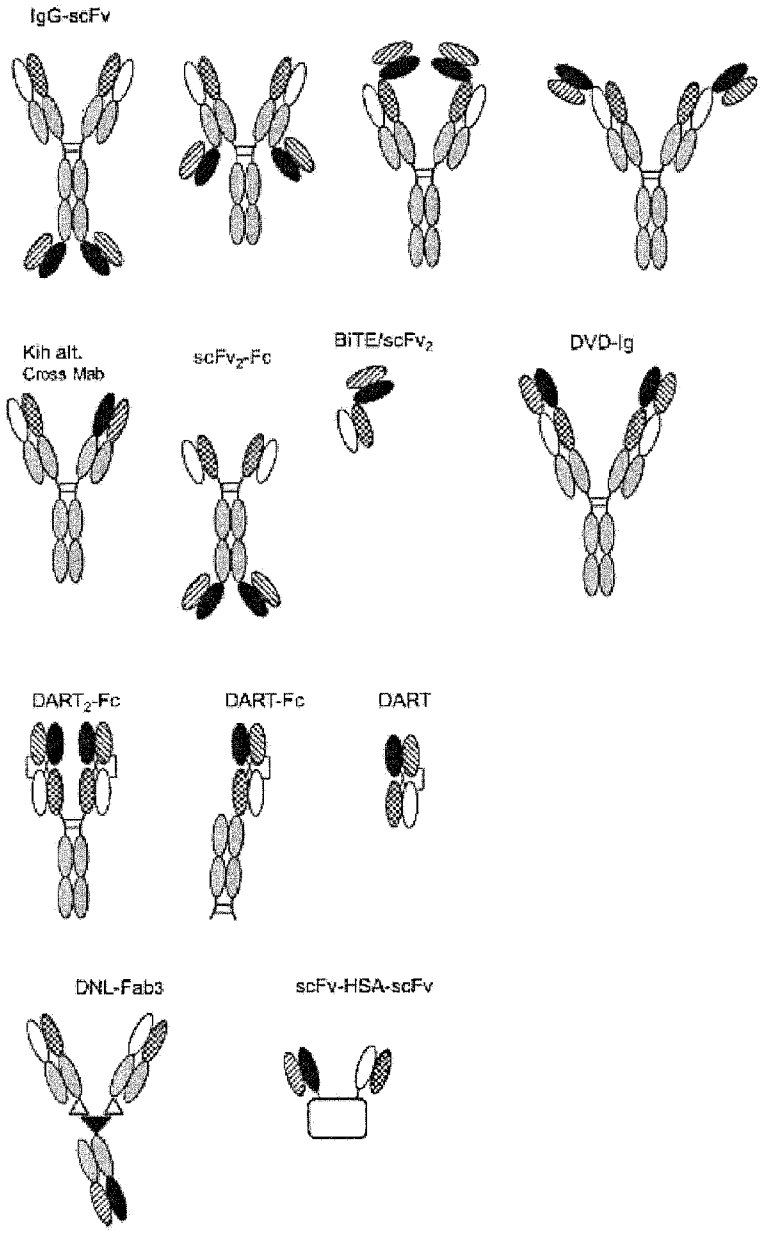


Figure 2

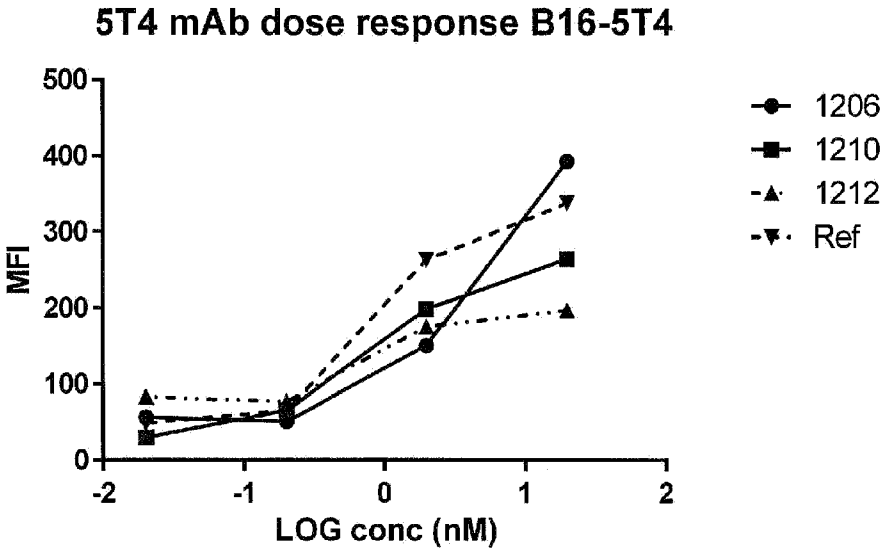
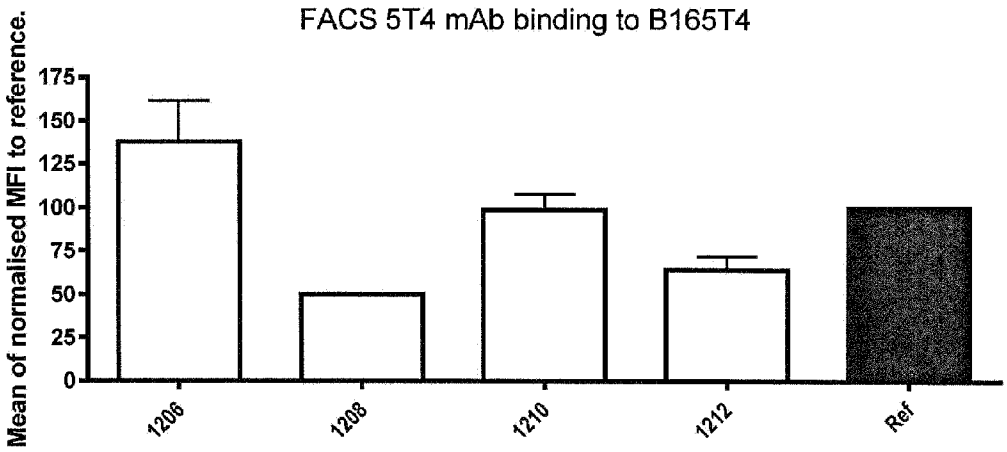

5T4 mAb dose response B16-5T4
MFI
500
400
300
200
100
0
-2
-1
0
1
2
LOG conc (nM)
1206
1210
1212
Ref

Figure 3



2.5 ug/ml

Figure 4

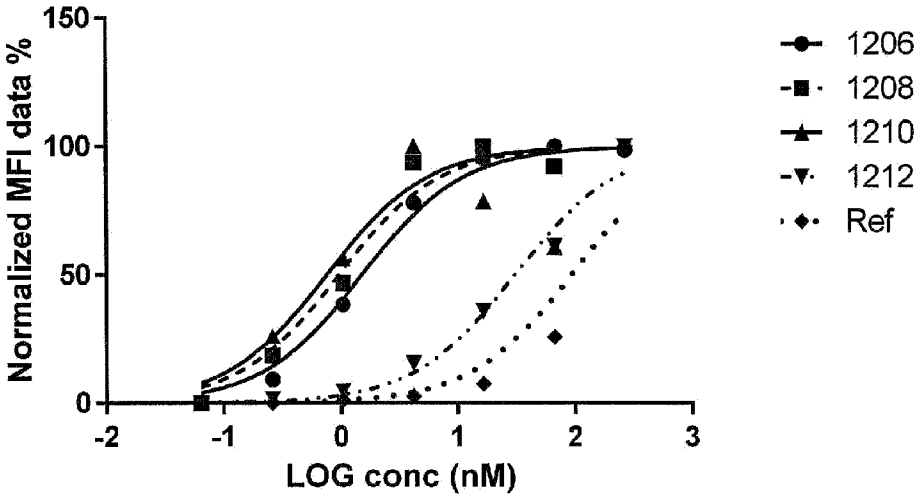

Normalized MFI data %
150
100
50
0
-2
-1
0
1
2
3
LOG conc (nM)
1206
1208
1210
1212
Ref

Figure 5

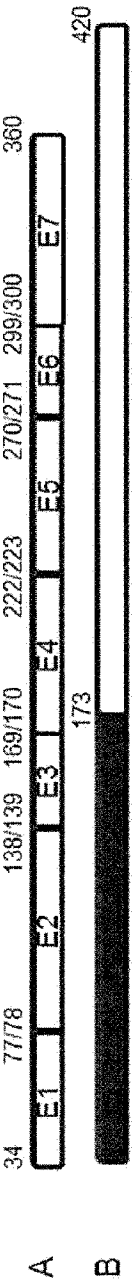

34
77/78
138/139
169/170
222/223
270/271
299/300
360
E1
E2
E3
E4
E5
E6
E7
173
420
A
B

Figure 6

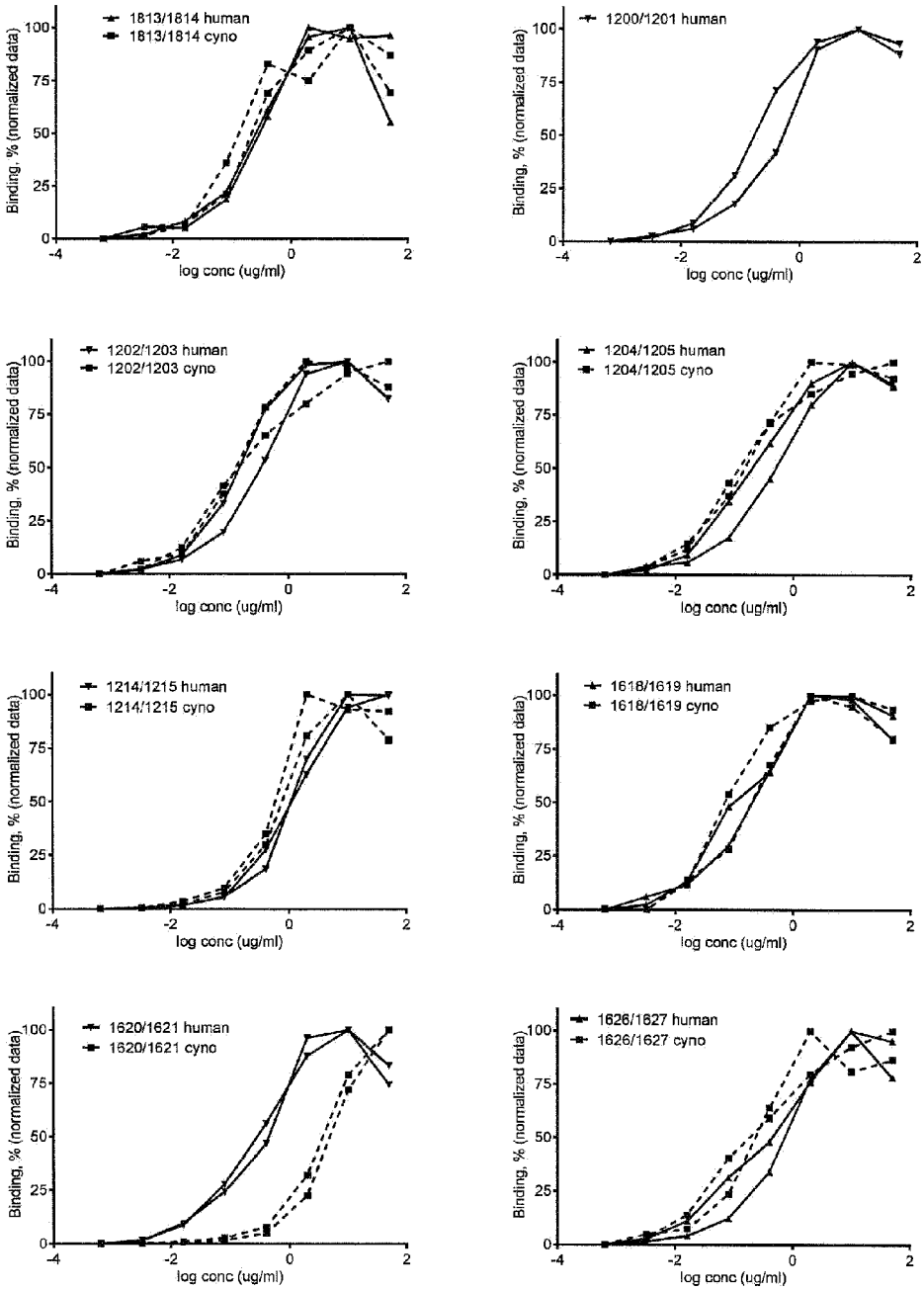

1813/1814 human
1813/1814 cyno
1200/1201 human
1202/1203 human
1202/1203 cyno
1204/1205 human
1204/1205 cyno
1214/1215 human
1214/1215 cyno
1618/1619 human
1618/1619 cyno
1620/1621 human
1620/1621 cyno
1626/1627 human
1626/1627 cyno
Binding, % (normalized data)
log conc (ug/ml)

Figure 7

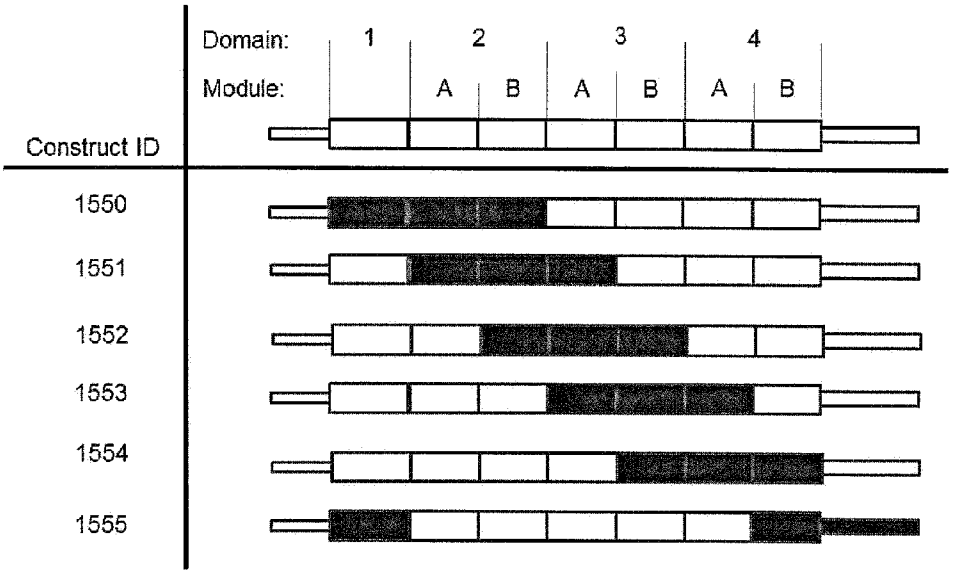

Domain:
1
2
3
4
Module:
A
B
A
B
A
B
Construct ID
1550
1551
1552
1553
1554
1555

Figure 8

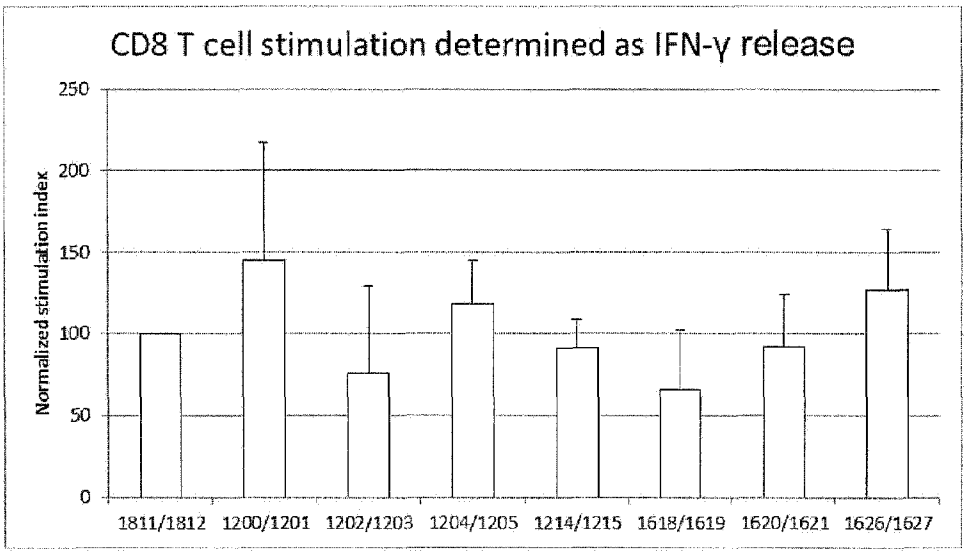

CD8 T cell stimulation determined as IFN-γ release
Normalized stimulation index
250
200
150
100
50
0
1811/1812
1200/1201
1202/1203
1204/1205
1214/1215
1618/1619
1620/1621
1626/1627

Figure 9

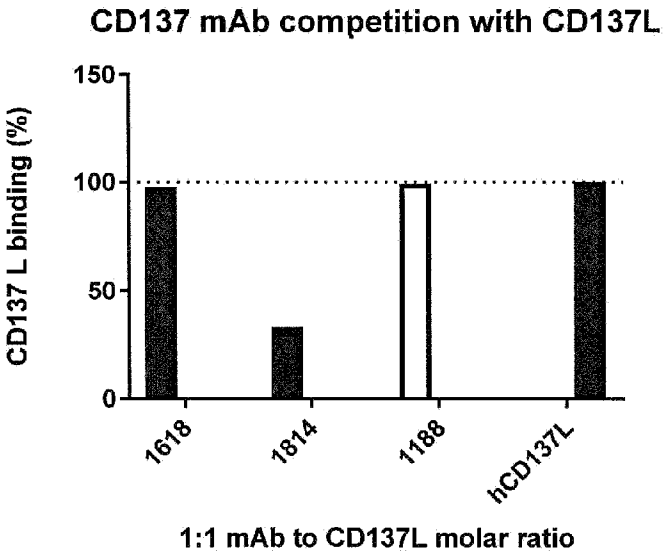

CD137 mAb competition with CD137L
CD137 L binding (%)
150
100
50
0
1618
1814
1188
hCD137L
1:1 mAb to CD137L molar ratio

Figure 10

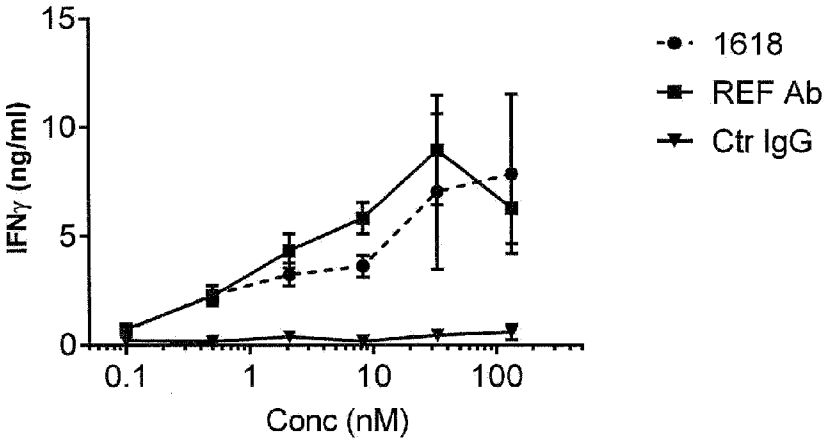


Figure 11

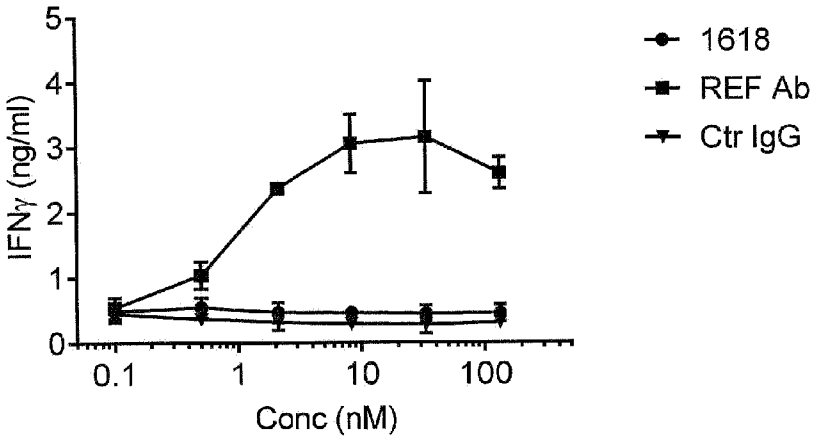


Figure 12

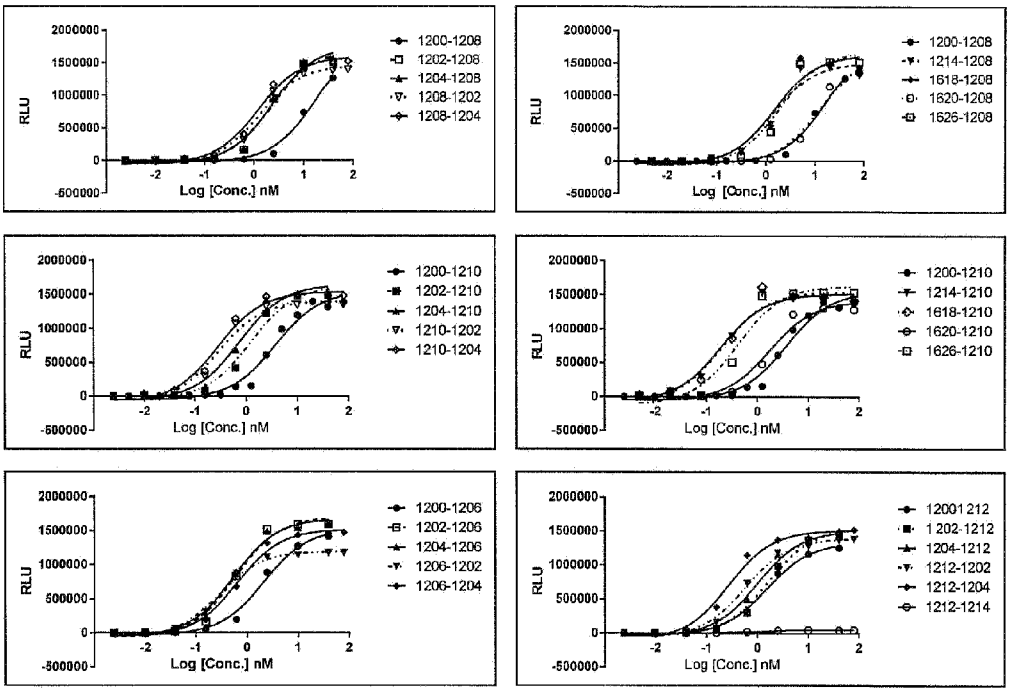

RLU
Log [Conc.] nM
1200-1208
1202-1208
1204-1208
1208-1202
1208-1204
1214-1208
1618-1208
1620-1208
1626-1208
1200-1210
1202-1210
1204-1210
1210-1202
1210-1204
1214-1210
1618-1210
1620-1210
1626-1210
1200-1206
1202-1206
1204-1206
1206-1202
1206-1204
12001212
1 202-1212
1204-1212
1212-1202
1212-1204
1212-1214

Figure 13

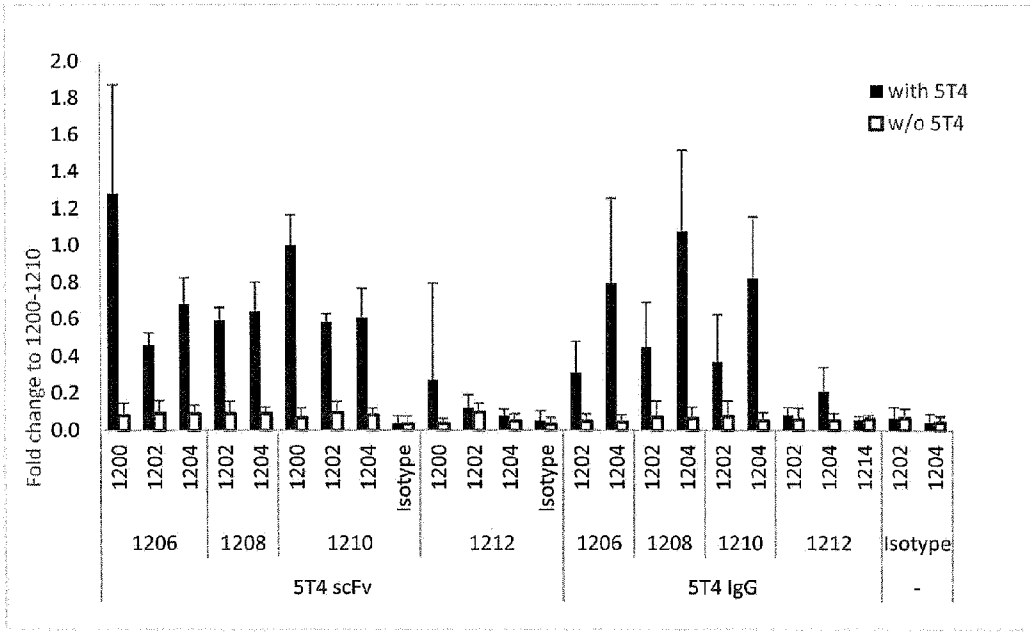

Fold change to 1200-1210
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
with 5T4
w/o 5T4
1200
1202
1204
1202
1204
1200
1202
1204
Isotype
1200
1202
1204
Isotype
1202
1204
1202
1204
1202
1204
1202
1204
1214
1202
1204
1206
1208
1210
1212
1206
1208
1210
1212
Isotype
5T4 scFv
5T4 IgG
-

Figure 14

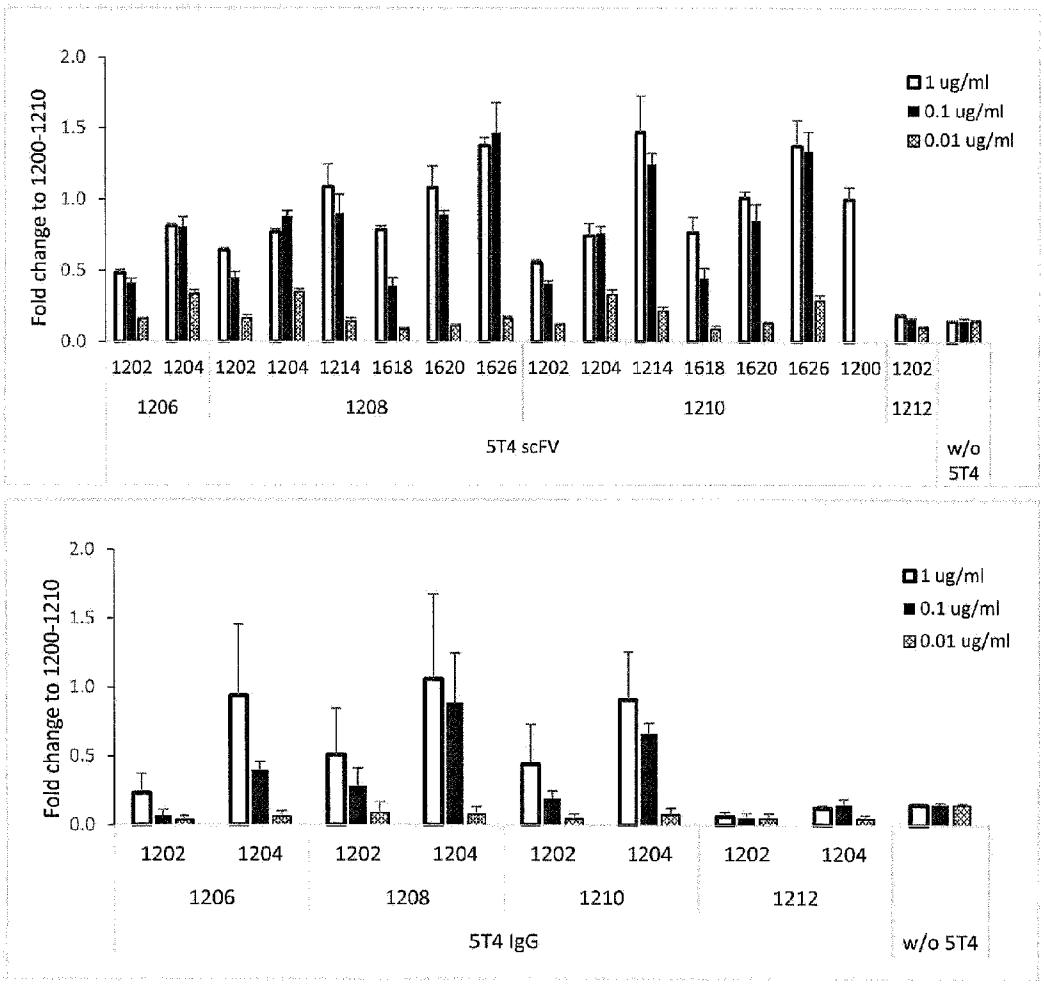

Fold change to 1200-1210
2.0
1.5
1.0
0.5
0.0
1 ug/ml
0.1 ug/ml
0.01 ug/ml
1202 1204 1202 1204 1214 1618 1620 1626 1202 1204 1214 1618 1620 1626 1200 1202
1206
1208
1210
1212
5T4 scFV
w/o 5T4
Fold change to 1200-1210
2.0
1.5
1.0
0.5
0.0
1 ug/ml
0.1 ug/ml
0.01 ug/ml
1202 1204 1202 1204 1202 1204 1202 1204
1206
1208
1210
1212
5T4 IgG
w/o 5T4

Figure 15

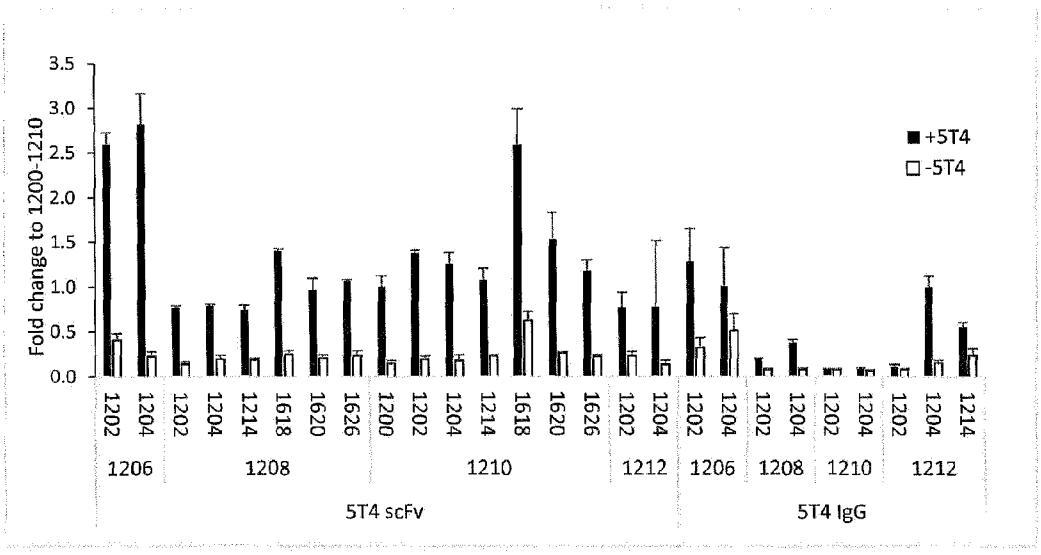

Fold change to 1200-1210
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
+5T4
-5T4
1202
1204
1202
1204
1214
1618
1620
1626
1200
1202
1204
1214
1618
1620
1626
1202
1204
1202
1204
1202
1204
1202
1204
1202
1204
1214
1206
1208
1210
1212
1206
1208
1210
1212
5T4 scFv
5T4 IgG

Figure 16

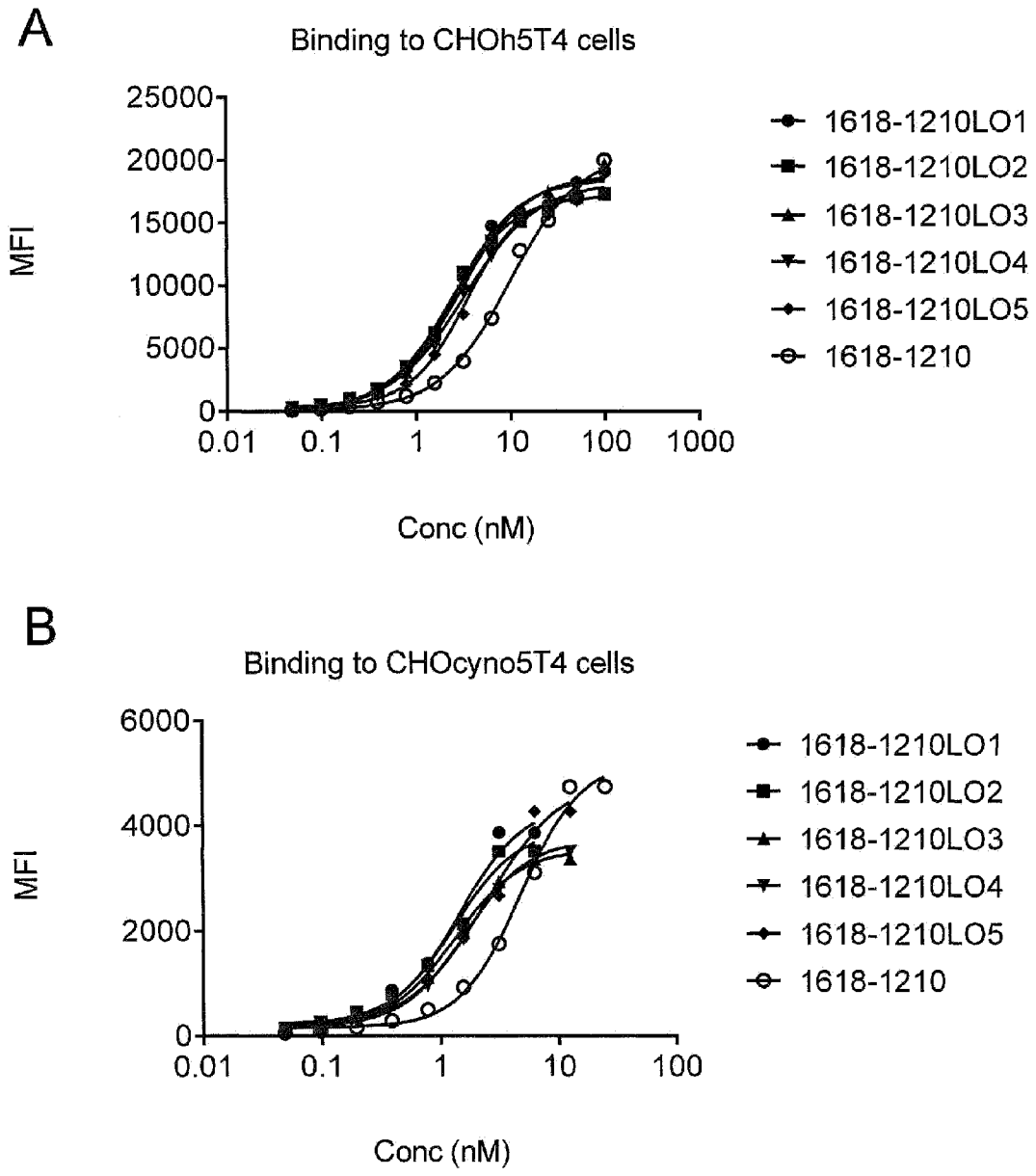
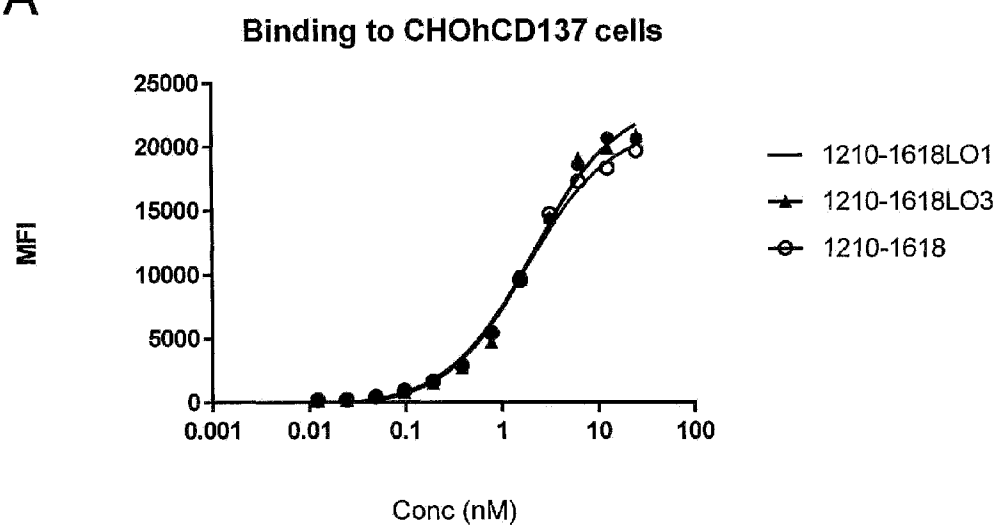

A
Binding to CHOh5T4 cells
MFI
25000
20000
15000
10000
5000
0
0.01
0.1
1
10
100
1000
Conc (nM)
1618-1210LO1
1618-1210LO2
1618-1210LO3
1618-1210LO4
1618-1210LO5
1618-1210
B
Binding to CHOcyno5T4 cells
MFI
6000
4000
2000
0
0.01
0.1
1
10
100
Conc (nM)
1618-1210LO1
1618-1210LO2
1618-1210LO3
1618-1210LO4
1618-1210LO5
1618-1210

Figure 17

A


Binding to CHOhCD137 cells
MFI
25000
20000
15000
10000
5000
0
0.001
0.01
0.1
1
10
100
Conc (nM)
1210-1618LO1
1210-1618LO3
1210-1618

B

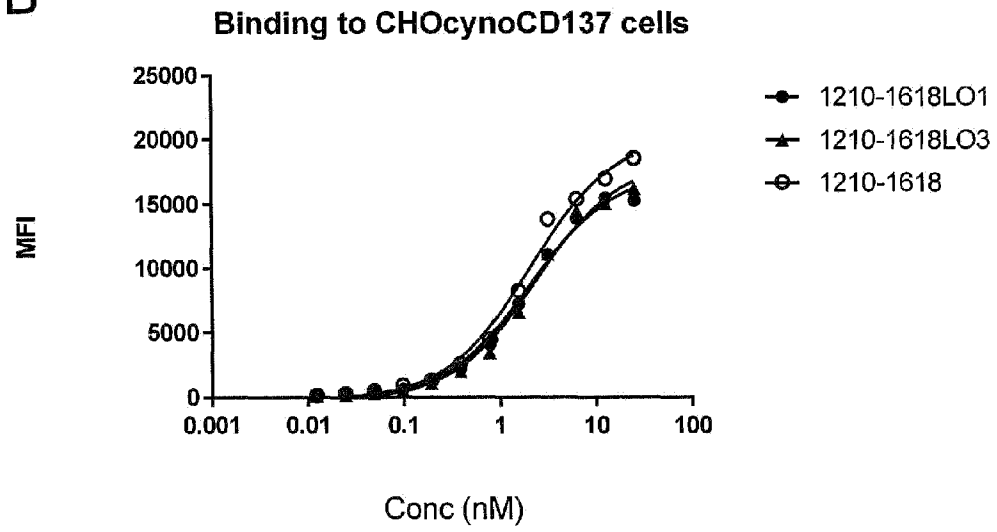

Binding to CHOcynoCD137 cells
MFI
25000
20000
15000
10000
5000
0
0.001
0.01
0.1
1
10
100
Conc (nM)
1210-1618LO1
1210-1618LO3
1210-1618

Figure 18

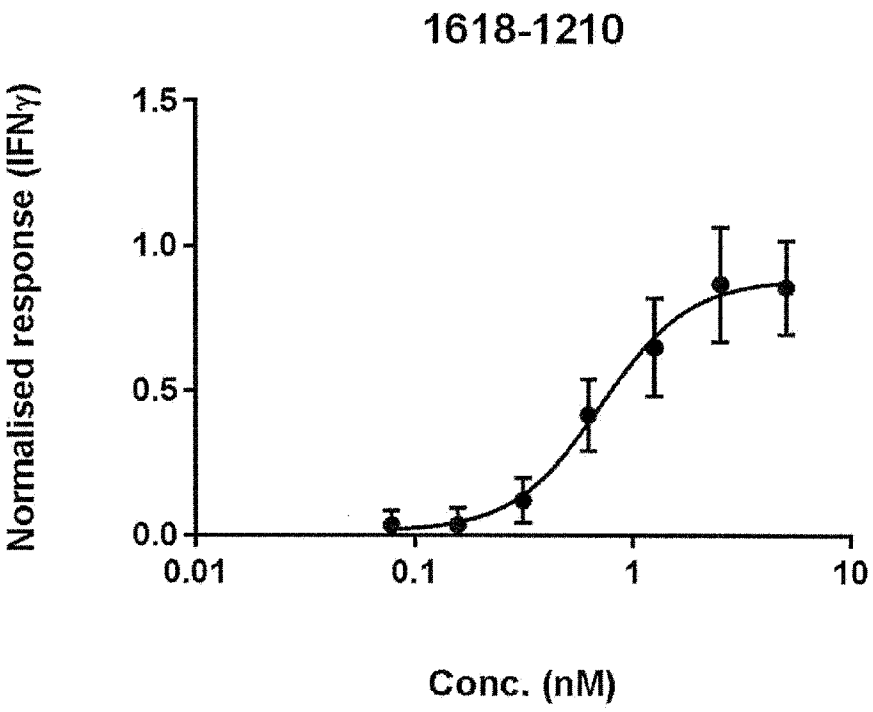

1618-1210
Normalised response (IFNγ)
1.5
1.0
0.5
0.0
0.01
0.1
1
10
Conc. (nM)

Figure 19

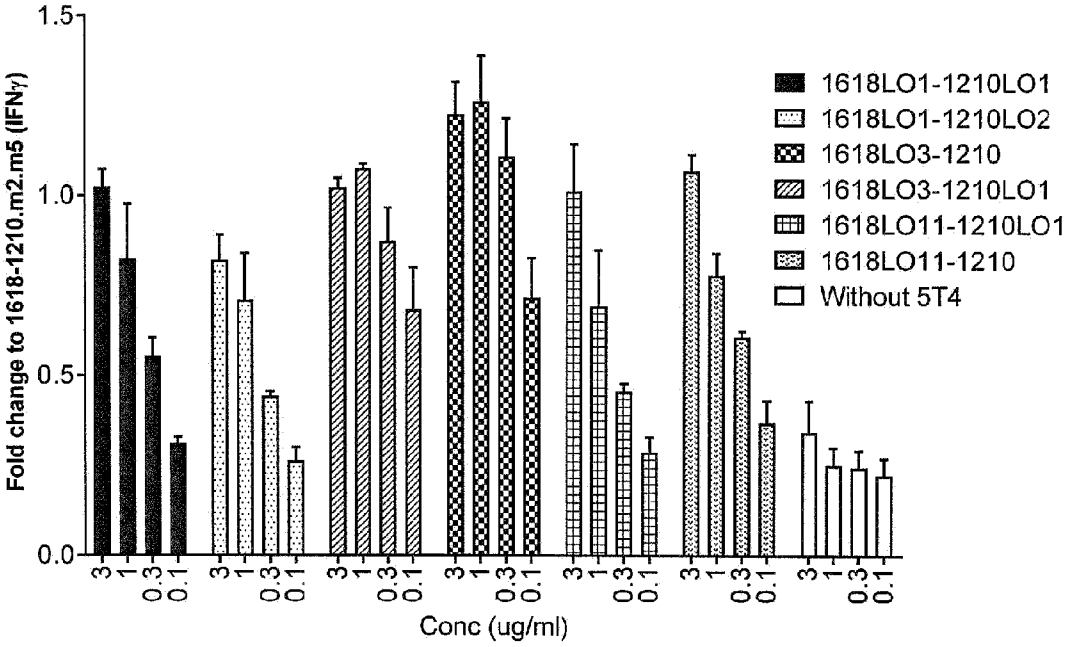

1.5
1.0
0.5
0.0
Fold change to 1618-1210.m2.m5 (IFNγ)
3
1
0.3
0.1
Conc (ug/ml)
1618LO1-1210LO1
1618LO1-1210LO2
1618LO3-1210
1618LO3-1210LO1
1618LO11-1210LO1
1618LO11-1210
Without 5T4

Figure 20

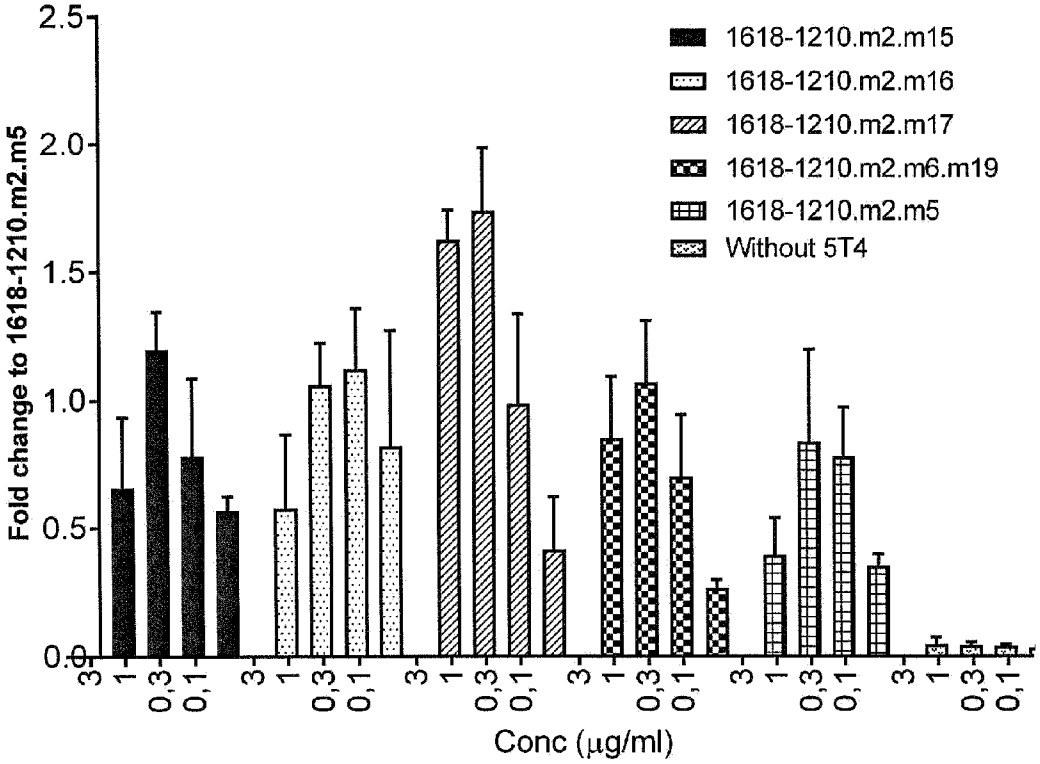

2.5
2.0
1.5
1.0
0.5
0.0
Fold change to 1618-1210.m2.m5
1618-1210.m2.m15
1618-1210.m2.m16
1618-1210.m2.m17
1618-1210.m2.m6.m19
1618-1210.m2.m5
Without 5T4
3
1
0,3
0,1
Conc (µg/ml)

Figure 21

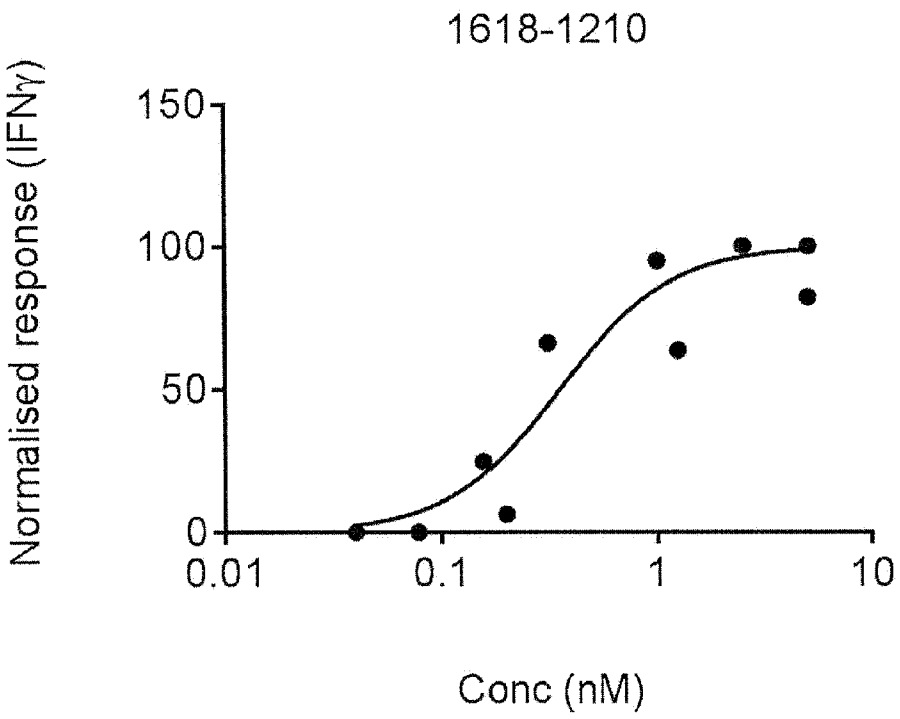

1618-1210
Normalised response (IFNγ)
150
100
50
0
0.01
0.1
1
10
Conc (nM)

Figure 22

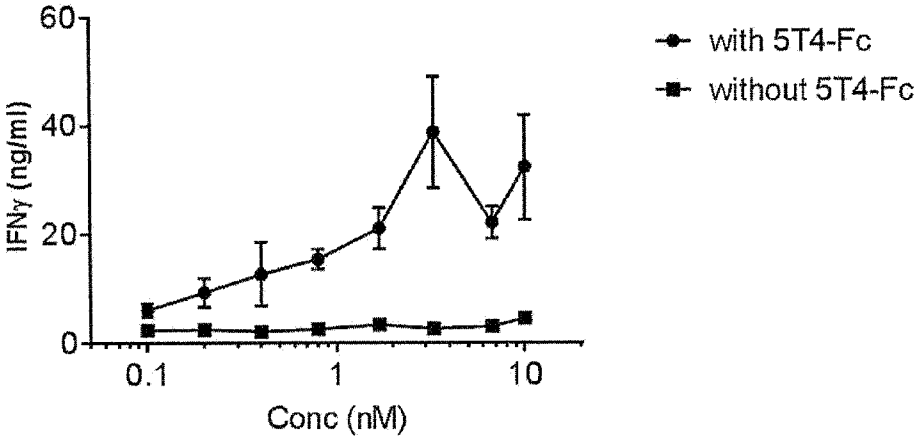


Figure 23

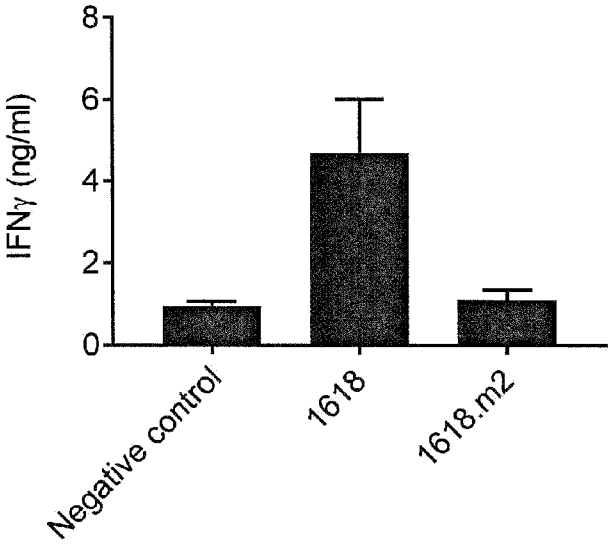


Figure 24

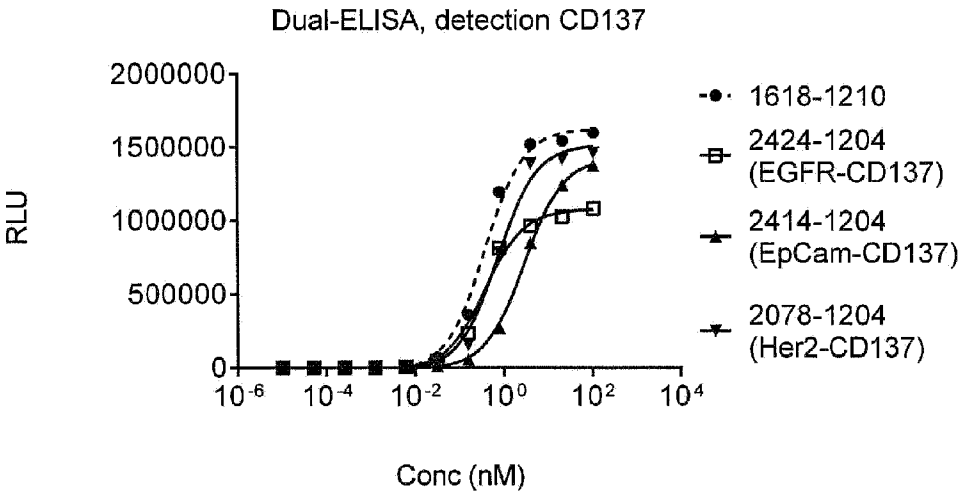
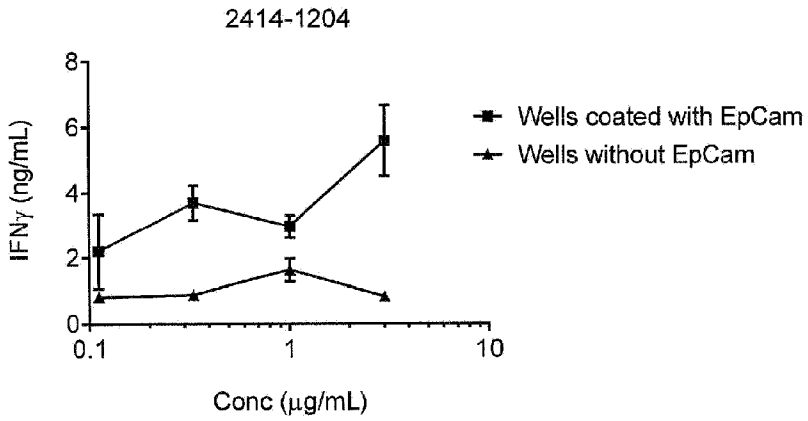
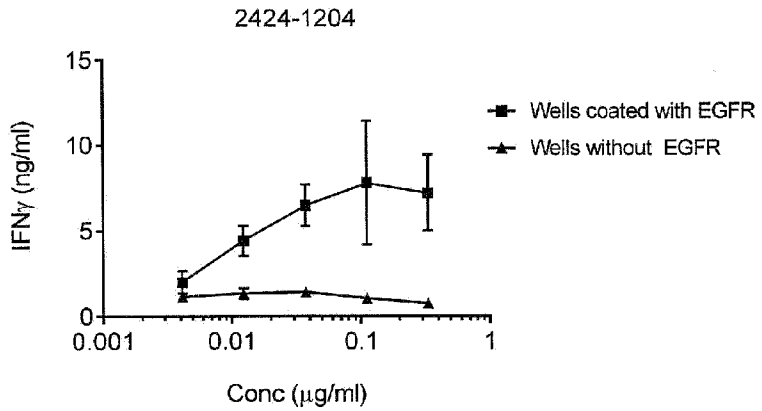

Dual-ELISA, detection CD137
RLU
2000000
1500000
1000000
500000
0
10-6
10-4
10-2
100
102
104
Conc (nM)
1618-1210
2424-1204
(EGFR-CD137)
2414-1204
(EpCam-CD137)
2078-1204
(Her2-CD137)

Figure 25

A



B



C

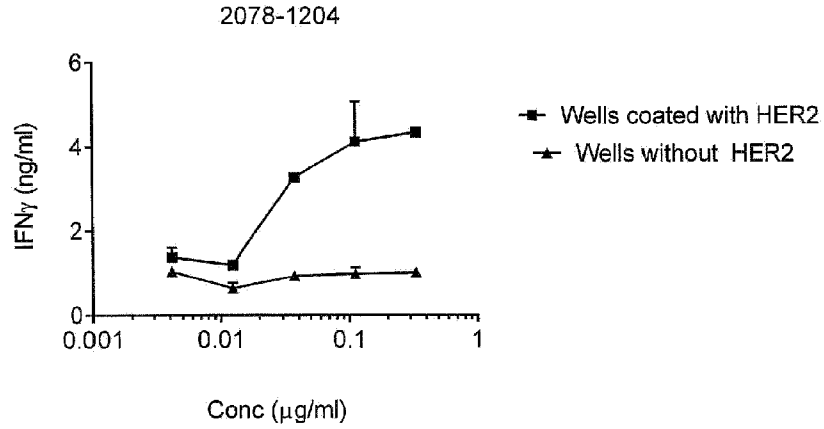


Figure 26

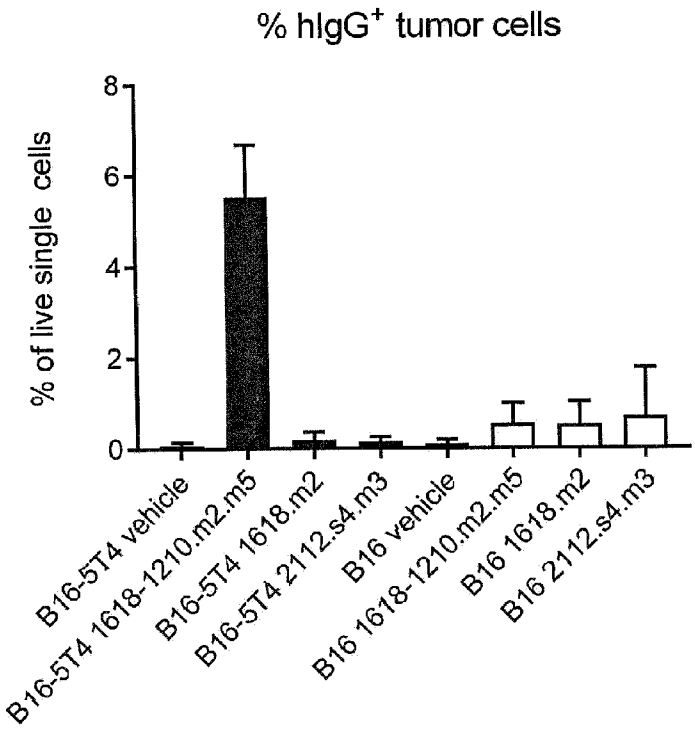
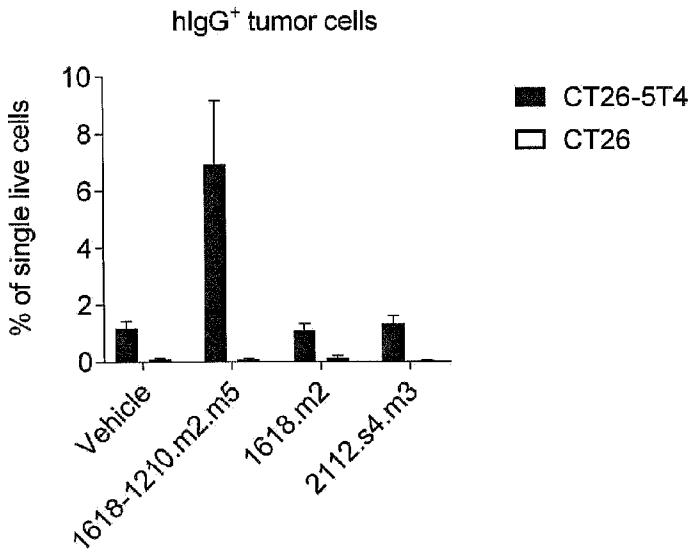

% hIgG+ tumor cells
% of live single cells
8
6
4
2
0
B16-5T4 vehicle
B16-5T4 1618-1210.m2.m5
B16-5T4 1618.m2
B16-5T4 2112.s4.m3
B16 vehicle
B16 1618-1210.m2.m5
B16 1618.m2
B16 2112.s4.m3

Figure 27

A


hIgG+ tumor cells
% of single live cells
10
8
6
4
2
0
CT26-5T4
CT26
Vehicle
1618-1210.m2.m5
1618.m2
2112.s4.m3

B

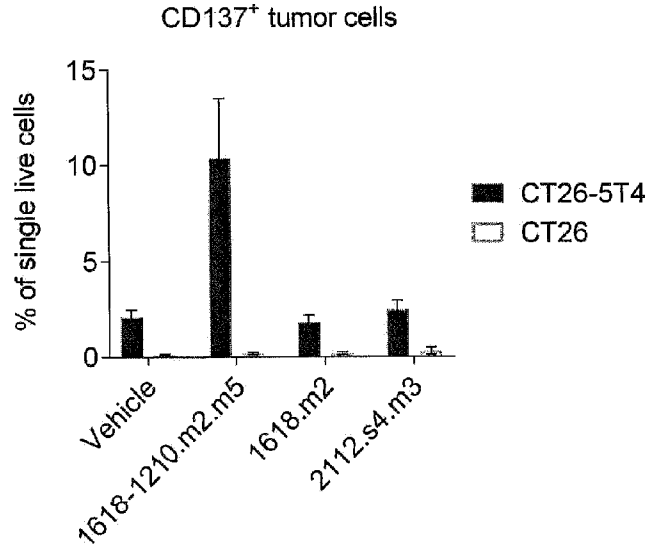
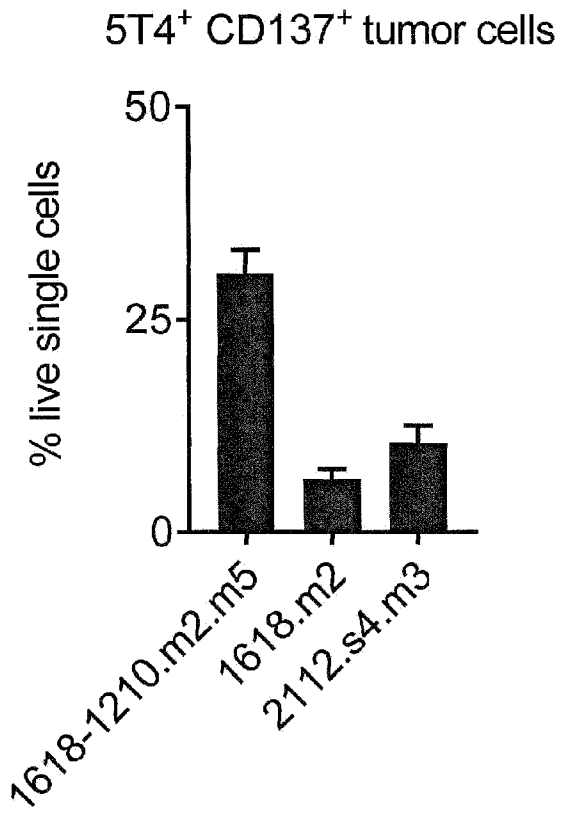

CD137+ tumor cells
% of single live cells
15
10
5
0
CT26-5T4
CT26
Vehicle
1618-1210.m2.m5
1618.m2
2112.s4.m3

Figure 28


5T4+ CD137+ tumor cells
% live single cells
50
25
0
1618-1210.m2.m5
1618.m2
2112.s4.m3