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(54) Title: TRIMERIC ANTIGEN BINDING MOLECULES SPECIFIC FOR A COSTIMULATORY TNF RECEPTOR

(57) Abstract: The invention relates to novel trimeric antigen binding molecules comprising three fusion polypeptides, each of the three fusion polypeptides comprising (a) a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO: 1, and (c) a moiety capable of specific binding to a target cell antigen, and to methods of producing these molecules and to methods of using the same.

Trimeric antigen binding molecules specific for a costimulatory TNF receptor

FIELD OF THE INVENTION

The invention relates to novel trimeric antigen binding molecules comprising three fusion polypeptides, each of the three fusion polypeptides comprising (a) a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain, in particular a trimerization domain derived from human cartilage matrix protein (hCMP, SEQ ID NO:1), and (c) a moiety capable of specific binding to a target cell antigen, and to methods of producing these molecules and to methods of using the same. The invention further relates to methods of producing these molecules and to methods of using the same.

BACKGROUND

Several members of the tumor necrosis factor receptor (TNFR) family function after initial T cell activation to sustain T cell responses and thus have pivotal roles in the organization and function of the immune system. CD27, 4-1BB (CD137), OX40 (CD134), HVEM, CD30, and GITR can have costimulatory effects on T cells, meaning that they sustain T-cell responses after initial T cell activation (Watts T.H. (2005) Annu. Rev. Immunol. 23, 23-68). The effects of these costimulatory TNFR family members can often be functionally, temporally, or spatially segregated from those of CD28 and from each other. The sequential and transient regulation of T cell activation/survival signals by different costimulators may function to allow longevity of the response while maintaining tight control of T cell survival. Depending on the disease condition, stimulation via costimulatory TNF family members can exacerbate or ameliorate disease. Despite these complexities, stimulation or blockade of TNFR family costimulators shows promise for several therapeutic applications, including cancer, infectious disease, transplantation, and autoimmunity.

Among several costimulatory molecules, the tumor necrosis factor (TNF) receptor family member OX40 (CD134) plays a key role in the survival and homeostasis of effector and memory T cells (Croft M. et al. (2009), Immunological Reviews 229, 173-191). OX40 (CD134) is expressed in several types of cells and regulates immune responses against infections, tumors and self-antigens and its expression has been demonstrated on the surface of T-cells, NKT-cells and NK-cells as well as neutrophils (Baumann R. et al. (2004), Eur. J. Immunol. 34, 2268-2275) and shown to be strictly inducible or strongly upregulated in response to various stimulatory signals. Functional activity of the molecule has been demonstrated in every OX40-expressing cell type suggesting complex regulation of OX40-mediated activity *in vivo*. Combined with T-

cell receptor triggering, OX40 engagement on T-cells by its natural ligand or agonistic antibodies leads to synergistic activation of the PI3K and NF κ B signalling pathways (Song J. et al. (2008) *J. Immunology* 180(11), 7240-7248). In turn, this results in enhanced proliferation, increased cytokine receptor and cytokine production and better survival of activated T-cells. In addition to its co-stimulatory activity in effector CD4⁺ or CD8⁺ T-cells, OX40 triggering has been recently shown to inhibit the development and immunosuppressive function of T regulatory cells. This effect is likely to be responsible, at least in part, for the enhancing activity of OX40 on anti-tumor or anti-microbial immune responses. Given that OX40 engagement can expand T-cell populations, promote cytokine secretion, and support T-cell memory, agonists including antibodies and soluble forms of the ligand OX40L have been used successfully in a variety of preclinical tumor models (Weinberg et al. (2000), *J. Immunol.* 164, 2160–2169).

4-1BB (CD137), a member of the TNF receptor superfamily, has been first identified as a molecule whose expression is induced by T-cell activation (Kwon Y.H. and Weissman S.M. (1989), *Proc. Natl. Acad. Sci. USA* 86, 1963-1967). Subsequent studies demonstrated expression of 4-1BB in T- and B-lymphocytes (Snell L.M. et al. (2011) *Immunol. Rev.* 244, 197-217 or Zhang X. et al. (2010), *J. Immunol.* 184, 787-795), NK-cells (Lin W. et al. (2008), *Blood* 112, 699-707, NKT-cells (Kim D.H. et al. (2008), *J. Immunol.* 180, 2062-2068), monocytes (Kienzle G. and von Kempis J. (2000), *Int. Immunol.* 12, 73-82, or Schwarz H. et al. (1995), *Blood* 85, 1043-1052), neutrophils (Heinisch I.V. et al. (2000), *Eur. J. Immunol.* 30, 3441-3446), mast (Nishimoto H. et al. (2005), *Blood* 106, 4241-4248), and dendritic cells as well as cells of non-hematopoietic origin such as endothelial and smooth muscle cells (Broll K. et al. (2001), *Am. J. Clin. Pathol.* 115, 543-549 or Olofsson P.S. et al. (2008), *Circulation* 117, 1292-1301). Expression of 4-1BB in different cell types is mostly inducible and driven by various stimulatory signals, such as T-cell receptor (TCR) or B-cell receptor triggering, as well as signaling induced through co-stimulatory molecules or receptors of pro-inflammatory cytokines (Diehl L. et al. (2002), *J. Immunol.* 168, 3755-3762; von Kempis J. et al. (1997), *Osteoarthritis Cartilage* 5, 394-406; Zhang X. et al. (2010), *J. Immunol.* 184, 787-795).

CD137 signaling is known to stimulate IFN γ secretion and proliferation of NK cells (Buechele C. et al. (2012), *Eur. J. Immunol.* 42, 737-748; Lin W. et al. (2008), *Blood* 112, 699-707; Melero I. et al. (1998), *Cell Immunol.* 190, 167-172) as well as to promote DC activation as indicated by their increased survival and capacity to secrete cytokines and upregulate co-stimulatory molecules (Choi B. K. et al. (2009), *J. Immunol.* 182, 4107-4115; Futagawa T. et al. (2002), *Int. Immunol.* 14, 275-286; Wilcox R. A. et al. (2002), *J. Immunol.* 168, 4262-4267). However, CD137 is best characterized as a co-stimulatory molecule which modulates TCR-induced activation in both the CD4⁺ and CD8⁺ subsets of T-cells. In combination with TCR triggering, agonistic 4-1BB-specific antibodies enhance proliferation of T-cells, stimulate lymphokine secretion and decrease sensitivity of T-lymphocytes to activation-induced cells

death (Snell L.M. et al. (2011) *Immunol. Rev.* 244, 197-217). In line with these co-stimulatory effects of 4-1BB antibodies on T-cells in vitro, their administration to tumor bearing mice leads to potent anti-tumor effects in many experimental tumor models (Melero I. et al. (1997), *Nat. Med.* 3, 682-685; Narazaki H. et al. (2010), *Blood* 115, 1941-1948). In vivo depletion
5 experiments demonstrated that CD8⁺ T-cells play the most critical role in anti-tumoral effect of 4-1BB-specific antibodies. However, depending on the tumor model or combination therapy, which includes anti-4-1BB, contributions of other types of cells such as DCs, NK-cells or CD4⁺ T-cells have been reported (Murillo O. et al. (2009), *Eur. J. Immunol.* 39, 2424-2436; Stagg J. et al. (2011), *Proc. Natl. Acad. Sci. USA* 108, 7142-7147).

10 In addition to their direct effects on different lymphocyte subsets, 4-1BB agonists can also induce infiltration and retention of activated T-cells in the tumor through 4-1BB-mediated upregulation of intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) on tumor vascular endothelium (Palazon A. et al. (2011), *Cancer Res.* 71, 801-811). 4-1BB triggering may also reverse the state of T-cell anergy induced by exposure to soluble
15 antigen that may contribute to disruption of immunological tolerance in the tumor micro-environment or during chronic infections (Wilcox R.A. et al. (2004), *Blood* 103, 177-184).

It appears that the immunomodulatory properties of 4-1BB agonistic antibodies in vivo require the presence of the wild type Fc-portion on the antibody molecule thereby implicating Fc-receptor binding as an important event required for the pharmacological activity of such
20 reagents as has been described for agonistic antibodies specific to other apoptosis-inducing or immunomodulatory members of the TNFR-superfamily (Li F. and Ravetch J.V. (2011), *Science* 333, 1030-1034; Teng M.W. et al. (2009), *J. Immunol.* 183, 1911-1920). However, systemic administration of 4-1BB-specific agonistic antibodies with the functionally active Fc domain also induces expansion of CD8⁺ T-cells associated with liver toxicity (Dubrot J. et al. (2010),
25 *Cancer Immunol. Immunother.* 59, 1223-1233) that is diminished or significantly ameliorated in the absence of functional Fc-receptors in mice. In human clinical trials (ClinicalTrials.gov, NCT00309023), Fc-competent 4-1BB agonistic antibodies (BMS-663513) administered once every three weeks for 12 weeks induced stabilization of the disease in patients with melanoma, ovarian or renal cell carcinoma. However, the same antibody given in another trial
30 (NCT00612664) caused grade 4 hepatitis leading to termination of the trial (Simeone E. and Ascierto P.A. (2012), *J. Immunotoxicology* 9, 241-247). Thus, there is a need for new generation agonists that should not only effectively engage 4-1BB on the surface of hematopoietic and endothelial cells but also be capable of achieving that through mechanisms other than binding to Fc-receptors in order to avoid uncontrollable side effects.

35 Several approaches have been described to generate artificial multimers with defined stoichiometries of recombinant antibodies or other proteins. WO 01/49866 discloses recombinant

fusion proteins comprising a TNF cytokine and a multimerization component, particularly proteins from the C1q protein family such as ACRP30 or a collectin. A disadvantage of these fusion proteins is, however, that the trimerization domain usually has a large molecular weight and/or that the trimerization is rather inefficient. WO 2007/014744, WO 2009/000538 and

5 Wyzgol et al. (2009) disclose fusion proteins comprising a TNF cytokine and a trimerization domain from the chicken protein tenascin. Biological activity could be strongly enhanced. However, a trimerization domain that is derived from a non-human origin, could have the disadvantage of causing immunogenicity reactions in the human body. Human cartilage matrix protein (huCMP) is a major extracellular matrix protein localized specifically in cartilage. Its C-

10 terminal region forms a three-stranded alpha-helical coiled-coil structure (Beck, 1996). WO 2017/025610 and WO 2015/183902 disclose GITR ligands and OX40 ligands, respectively, that are fused to a trimerization domain which is connected to a Fc domain. However, these molecules lack the "tumor-targeting" through a moiety capable of binding to tumor-specific target and thus could lead to unspecific immune reactions.

15 The available pre-clinical and clinical data clearly demonstrate that there is a high clinical need for effective agonists of costimulatory TNFR family members such as OX40 and 4-1BB that are able to induce and enhance effective endogenous immune responses to cancer. However, almost never are the effects limited to a single cell type or acting via a single mechanism and studies designed to elucidate inter- and intracellular signaling mechanisms have revealed

20 increasing levels of complexity. Thus, there is a need of "targeted" agonists that preferably act on a single cell type. The antigen binding molecules of the invention combine a moiety capable of preferred binding to tumor-specific or tumor-associated targets with a moiety capable of agonistic binding to costimulatory TNF receptors. The antigen binding molecules of this invention may be able to trigger TNF receptors not only effectively, but also very selectively at

25 the desired site thereby reducing undesirable side effects.

SUMMARY OF THE INVENTION

The present invention provides a trimeric antigen binding molecule comprising three fusion polypeptides, each of the three fusion polypeptides comprising

(a) a moiety capable of specific binding to a costimulatory TNF receptor family member,

30 (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1, and

(c) a moiety capable of specific binding to a target cell antigen.

The novel trimeric antigen binding molecules of the present invention are stable, as the fusion polypeptides associate with each other through the huCMP trimerization domain. The

35 stable antigen binding molecule trimers are able to trigger TNF receptors highly effectively due

to their trimeric structure, but also very selectively at the site where the target cell antigen is expressed, due to their binding capability towards a target cell antigen. Side effects may therefore be drastically reduced.

5 In some embodiments, the trimeric antigen binding molecule comprises three fusion polypeptides, each of the three fusion polypeptides comprising a trimerization domain comprising an amino acid sequence having at least 95% identity to SEQ ID NO:2. More particularly, the trimerization domain comprises the amino acid sequence of SEQ ID NO:2.

10 In some embodiments, the three fusion polypeptides are linked by disulphide bonds. In some embodiments, the three fusion polypeptides are linked by disulphide bonds formed between the trimerization domains of the fusion polypeptides.

In some embodiments, the costimulatory TNF receptor family member is selected from OX40 and 4-1BB.

15 In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:3.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety comprises a heavy chain variable domain (VH) comprising

20 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5,

(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and

25 (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14,

and a light chain variable domain (VL) comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17,

30 (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25 and SEQ ID NO:26.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises a heavy chain variable domain (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO: 29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37 and SEQ ID NO:39 and a light chain variable domain (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO: 30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 and SEQ ID NO:40.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises

- (i) a heavy chain variable domain (VH) comprising the amino acid sequence of SEQ ID NO:27 and light chain variable domain (VL) comprising the amino acid sequence of SEQ ID NO:28,
- (ii) a VH comprising the amino acid sequence of SEQ ID NO:29 and a VL comprising the amino acid sequence of SEQ ID NO:30,
- (iii) a VH comprising the amino acid sequence of SEQ ID NO:31 and a VL comprising the amino acid sequence of SEQ ID NO:32,
- (iv) a VH comprising the amino acid sequence of SEQ ID NO:33 and a VL comprising the amino acid sequence of SEQ ID NO:34,
- (v) a VH comprising the amino acid sequence of SEQ ID NO:35 and a VL comprising the amino acid sequence of SEQ ID NO:36,
- (vi) a VH comprising the amino acid sequence of SEQ ID NO:37 and a VL comprising the amino acid sequence of SEQ ID NO:38, or
- (vii) a VH comprising the amino acid sequence of SEQ ID NO:39 and a VL comprising the amino acid sequence of SEQ ID NO:40.

In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:41.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety comprises a VH comprising

- (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:42 and SEQ ID NO:43,
- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50, and a VL comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:51 and SEQ ID NO:52,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:53 and SEQ ID NO:54, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58 and SEQ ID NO:59.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66 and SEQ ID NO:68 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67 and SEQ ID NO:69.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:60 and a VL comprising the amino acid sequence of SEQ ID NO:61,

(ii) a VH comprising the amino acid sequence of SEQ ID NO:62 and a VL comprising the amino acid sequence of SEQ ID NO:63,

(iii) a VH comprising the amino acid sequence of SEQ ID NO:64 and a VL comprising the amino acid sequence of SEQ ID NO:65,

(iv) a VH comprising the amino acid sequence of SEQ ID NO:66 and a VL comprising the amino acid sequence of SEQ ID NO:67, or

(v) a VH comprising the amino acid sequence of SEQ ID NO:68 and a VL comprising the amino acid sequence of SEQ ID NO:69.

In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member is fused at the C-terminal amino acid to the N-terminal amino acid of the trimerization domain, optionally through a peptide linker.

In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member is a Fab fragment or a scFv.

In some embodiments, the target cell antigen is selected from the group consisting of Fibroblast Activation Protein (FAP), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), Carcinoembryonic Antigen (CEA), CD19, CD20 and CD33. In some embodiments, the target cell antigen is FAP or CEA. In particular, the target cell antigen is FAP.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising

(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:70 and SEQ ID NO:76,

(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:71 and SEQ ID NO:77, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:72 and SEQ ID NO:78,

and a VL comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:73 and SEQ ID NO:79,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:74 and SEQ ID NO:80, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75 and SEQ ID NO:81.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a heavy chain variable domain (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:82 and SEQ ID NO:84 and a light chain variable domain (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:83 and SEQ ID NO:85.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:82 and a VL comprising the amino acid sequence of SEQ ID NO:83, or

(ii) a VH comprising the amino acid sequence of SEQ ID NO:84 and a VL comprising the amino acid sequence of SEQ ID NO:85.

In some embodiments, the moiety capable of specific binding to a target cell antigen is fused at the N-terminal amino acid to the C-terminal amino acid of the trimerization domain, optionally through a peptide linker.

In some embodiments, three fusion polypeptides are identical.

5 The present invention also provides a fusion polypeptide comprising (a) a VH and/or VL of a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) comprising the amino acid sequence of SEQ ID NO:2, wherein the trimerization domain is capable of mediating stable association of the fusion polypeptide with two further such fusion polypeptides and (c) a
10 VH and/or VL of a moiety capable of specific binding to a target cell antigen.

In some embodiments, the fusion polypeptide comprises a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising

- 15 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:70 and SEQ ID NO:76,
- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:71 and SEQ ID NO:77, and
- (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:72 and SEQ ID NO:78,
- 20 and a VL comprising
- (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:73 and SEQ ID NO:79,
- (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:74 and SEQ ID NO:80, and
- 25 (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75 and SEQ ID NO:81.

In some embodiments, the fusion polypeptide comprises a VH of a moiety capable of specific binding to OX40, wherein the VH comprises

- 30 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5,
- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and
- (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID
35 NO:13 and SEQ ID NO:14.

In some embodiments, the fusion polypeptide comprises a VH of a moiety capable of specific binding to 4-1BB, wherein the VH comprises

(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:42 and SEQ ID NO:43,

5 (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50.

10 The present invention also provides a polynucleotide encoding the trimeric antigen binding molecule of the invention, or the fusion polypeptide of the invention.

The present invention also provides a vector, e.g. an expression vector, comprising the polynucleotide of the invention.

15 The present invention also provides a host cell comprising the polynucleotide of the invention or the expression vector of the invention. In some embodiments the host cell is a eukaryotic cell, particularly a mammalian cell.

20 The present invention also provides a method of producing a trimeric antigen binding molecule, comprising culturing the host cell of the invention under conditions suitable for the expression of the trimeric antigen binding molecule, and isolating the trimeric antigen binding molecule. The invention also encompasses a trimeric antigen binding molecule produced by the method of the invention.

The present invention also provides a pharmaceutical composition comprising the trimeric antigen binding molecule of the invention and at least one pharmaceutically acceptable excipient.

The present invention also provides the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

25 The present invention also provides the trimeric antigen binding molecule as described herein before, or the pharmaceutical composition of the invention, for use

(i) in stimulating T cell response,

(ii) in supporting survival of activated T cells,

(iii) in the treatment of infections,

30 (iv) in the treatment of cancer,

(v) in delaying progression of cancer, or

(vi) in prolonging the survival of a patient suffering from cancer.

The present invention also provides the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in the treatment of a disease in an individual in need thereof. Also provided is the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in the treatment of cancer.

5 The present invention also provides the use of the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, in the manufacture of a disease in an individual in need thereof, in particular for the manufacture of a medicament for the treatment of cancer.

10 The present invention also provides a method of treating a disease in an individual, comprising administering to the individual a therapeutically effective amount of a composition comprising the trimeric antigen binding molecule of the invention in a pharmaceutically acceptable form. Also provided is a method of treating an individual having cancer, said method comprising administering to the individual an effective amount of the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention.

15 The present invention also provides the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in up-regulating or prolonging cytotoxic T cell activity. Also provided is the use of the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, in the manufacture of a medicament for up-regulating or prolonging cytotoxic T cell activity. Also provided is a method
20 of up-regulating or prolonging cytotoxic T cell activity in an individual having cancer, comprising administering to the individual an effective amount of the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention.

In some embodiments in accordance with various aspects of the present invention the individual is a mammal, particularly a human.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the monomeric form of Fc-linked TNF receptor antigen that was used for the preparation of TNF receptor antibodies.

Figure 2A shows a schematic representation of the trimeric, bispecific, antigen binding molecules comprising three anti-OX40 Fab, and three scFv capable of specific binding to FAP.
30 **Figure 2B** shows the trimeric, bispecific antigen binding molecules comprising three anti-4-1BB Fab, and three scFv capable of specific binding to FAP.

Figure 3A shows a schematic representation of the set up of the surface plasmon resonance assays for simultaneous binding of human OX40 and human FAP by the trimeric,

bispecific anti-OX40, anti-FAP antigen binding molecules. **Figure 3B** shows a schematic representation of the set up of the surface plasmon resonance assays for simultaneous binding of human 4-1BB and human FAP by the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules.

5 **Figures 4A to 4E** show the binding of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules to recombinant OX40 Fc (kih) receptor and human FAP protein, as assessed by surface plasmon resonance. **Figure 4A** shows binding of molecules comprising anti-OX40 clone 8H9, **Figure 4B** shows binding of molecules comprising anti-OX40 clone 1G4, **Figure 4C** shows binding of molecules comprising anti-OX40 clone 49B4, **Figure 4D** shows binding of
10 molecules comprising anti-OX40 clone 21H4, and **Figure 4E** shows binding of molecules comprising anti-OX40 clone CLC-563.

Figures 5A to 5C show the binding of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules to resting and activated human CD4+ and CD8+ T cells. **Figure 5A** shows binding to resting CD4+ T cells, **Figure 5B** shows binding to activated CD4+ T cells, **Figure 5C**
15 shows binding to resting CD8+ T cells, and **Figure 5D** shows binding to activated CD8+ T cells. Binding is shown as the median of fluorescence intensity (MFI) of FITC conjugated anti-human IgG F(ab')₂-fragment-specific goat IgG F(ab')₂ fragment, which is used as secondary detection antibody. MFI was measured by flow cytometry and baseline corrected by subtracting the MFI of the blank control. The x-axis shows the concentration of the antigen binding molecules. All
20 OX40 clones bind to activated, OX40 expressing human CD4+ T cells, and to a lower extent to activated human CD8+ T cells. OX40 is not expressed on resting human PBMCs (**Figure 5A** and **5C**). After activation, OX40 is up-regulated on CD4+ and CD8+ T cells (**Figure 5B** and **5D**). OX40 expression on human CD8+ T cells is lower than on CD4+ T cells. The clones vary in the strength of binding (EC50 values as well as signal strength) to OX40 positive cells.

25 **Figures 6A and 6B** show the binding of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules to tumor cells which do not express FAP (FAP-), or which express FAP (FAP+). **Figure 6A** shows binding to CHO cells, which are negative for FAP. **Figure 6B** shows binding to WM266-4 cells, which express high levels of human fibroblast activation protein (huFAP). Binding is shown as the median of fluorescence intensity (MFI) of FITC
30 conjugated anti-human IgG F(ab')₂-fragment-specific goat IgG F(ab')₂ fragment, which is used as secondary detection antibody. MFI was measured by flow cytometry. The x-axis shows the concentration of antibody constructs. The molecules are shown to bind to FAP-expressing WM266-4 cells (**Figure 6B**), but not to FAP-negative CHO cells (**Figure 6A**).

Figures 7A to 7E show activation of NFκB by the trimeric, bispecific anti-OX40, anti-
35 FAP antigen binding molecules, in the presence or absence of crosslinking by FAP-positive cells.

Figure 7A shows NFκB activation by molecules comprising anti-OX40 clone 49B4, **Figure 7B** shows NFκB activation by molecules comprising anti-OX40 clone 1G4, **Figure 7C** shows NFκB activation by molecules comprising anti-OX40 clone CLC-563, **Figure 7D** shows NFκB activation by molecules comprising anti-OX40 clone 8H9, and **Figure 7E** shows NFκB activation by molecules comprising anti-OX40 clone 21H4. Open triangles show activation of NFκB in the absence of hyper-crosslinking, and filled circles show activation of NFκB following hyper-crosslinking by culture in the presence of FAP-expressing NIH/3T3-huFAP clone 39 cells (2:1 ratio of FAP⁺ tumor cells to reporter cells). NF-κB-mediated luciferase activity was characterized by plotting the units of released light (URL), measured during 0.5 s, versus the concentration of the antigen binding molecule (in nM). URLs are emitted due to luciferase-mediated oxidation of luciferin to oxyluciferin. The values were baseline-corrected by subtracting the URLs for a 'blank control' condition. All of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules induced NFκB activation in OX40⁺ HeLa reporter cells in the absence of FAP-positive cells, and addition of FAP-positive cells resulted in a strong increase in NFκB activation.

Figures 8A to 8D show activation of NFκB by the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules, in the presence or absence of crosslinking by FAP-positive cells. **Figure 8A** shows activation of NFκB by molecules comprising anti-OX40 clones 49B4, 1G4, CLC-356, 21H4 and 8H9, or DP47 huIgG1 P329GLALA (isotype control), following hyper-crosslinking by culture in the presence of FAP-expressing NIH/3T3-huFAP clone 39 cells (2:1 ratio of FAP⁺ tumor cells to reporter cells), **Figure 8B** shows activation of NFκB by molecules comprising anti-OX40 clones 49B4, 1G4, CLC-356, 21H4 and 8H9 in the absence of hyper-crosslinking. NF-κB-mediated luciferase activity was characterized by plotting the units of released light (URL), measured during 0.5 s, versus the concentration of the antigen binding molecule (in nM). URLs are emitted due to luciferase-mediated oxidation of luciferin to oxyluciferin. The values are baseline-corrected by subtracting the URLs for a 'blank control' condition. Limited, dose-dependent NFκB activation observed in the absence of FAP⁺ cells, in line with the observation that the trimeric OX40L naturally engages three OX40 receptors on the cell surface to form the basic signaling unit. Hyper-crosslinking of anti-OX40 antibodies by FAP expressing NIH/3T3huFAP cells strongly increased the induction of NFκB-mediated luciferase-activation, in a concentration-dependent manner. Full agonism of the OX40 axis may only be achieved when at least a hexameric OX40 signaling unit is assembled, which could be facilitated by FAP mediated immobilization of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules. However, with increasing concentration a drop in bioactivity was observed after an initial increase; it may be that at high concentration, efficient hyper-crosslinking is limited by insufficient FAP molecules at the cell surface, or by steric hindrance. NFκB activation then drops back to the levels observed in the absence of hyper-crosslinking.

Figure 8C shows NF κ B activation in the presence of hyper-crosslinking (**Figure 8A**) minus NF κ B activation in the absence of hyper-crosslinking (**Figure 8B**). **Figure 8D** shows the data of **Figure 8C** represented as area under the curve (AUC).

Figures 9A to 9D show rescue of suboptimal TCR restimulation of preactivated CD4 T cells with plate-immobilized trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules. Suboptimally PHA-L pre-activated CFSE-labeled human CD4 T cells were cultured for four days on plates pre-coated with mouse IgG Fc γ -specific antibodies (2 ug/mL), human IgG Fab specific antibodies (2 ug/mL), mouse anti-human CD3 antibodies (clone OKT3, [3 ng/mL]) and titrated trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules, or DP47 huIgG1 P329GLALA (isotype control). Cells were stained with a combination of fluorochrome-labeled mouse anti-human TIM-3 (clone F38-2E2, Biolegend, Ca.No.345008) and anti-CD127 (clone A019D5, Biolegend, Ca.No.351234), and analysed by flow cytometry. Values were baseline-corrected to values for samples containing only the plate-immobilized anti-human CD3. **Figure 9A** shows the number of events, **Figure 9B** shows the percentage of proliferating (i.e. CFSE_{low}) cells, **Figure 9C** shows the percentage of effector T cells (i.e. CD127_{low}) and **Figure 9D** shows the percentage of cells with an activated phenotype (Tim-3 positive cells). All of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules were able to rescue suboptimal TCR stimulation of preactivated, OX40⁺ CD4 T cells when coated to plate.

Figure 10 shows the EC₅₀ values of the plate-immobilized trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules for rescuing suboptimal TCR stimulation, as calculated from the data of **Figure 9A to 9D**. Event count, the percentage of proliferating (CFSE_{low}) cells, the percentage of CD127_{low} and Tim-3 positive cells at day 4 were plotted against the antigen binding molecule concentration, and EC₅₀ values as measure for agonistic strength were calculated using the inbuilt sigmoidal dose response quotation in Prism4 (GraphPad Software, USA). All of the trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules were able to rescue suboptimal TCR stimulation of preactivated, OX40⁺ CD4 T cells when plate-immobilised, and potency was clone dependent.

Figures 11A to 11D show rescue of suboptimal TCR restimulation of preactivated CD4 T cells with plate-immobilized trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules, in the presence or absence of crosslinking by FAP-positive cells. CFSE-labeled human PBMCs were activated with anti-human CD3 antibodies (clone V9, human IgG1; 2 nM) and trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules or non-targeted trivalent OX40 antigen binding molecules, at the indicated concentrations, for four days. Cells were subsequently surface-stained with fluorescent dye-conjugated anti-human CD4 (clone RPA-T4, BioLegend, Cat.-No. 300532), anti-CD8 (clone RPa-T8, BioLegend, Cat.-No. 3010441) and anti-CD25 (clone M-A251, BioLegend, Cat.-No. 356112), and analysed by flow cytometry.

Values were baseline-corrected to values for samples containing only the anti-human CD3 (clone V9, huIgG1), resting human PBMC and NIH/3T3-huFAP clone 39. **Figures 11A and 11C** show the MFI of CD25 (as a marker of activation) on vital CD4⁺ T cells. **Figures 11B and 11D** show the percentage of CD4⁺ T cells which were CD4⁺ CD25⁺ T cells. Only

5 constructs containing FAP binding moiety were able to rescue suboptimal TCR stimulation of preactivated, OX40⁺ CD4 T cells when crosslinking was provided by FAP positive cells. The high affinity clone 8H9 (**Figures 11A and 11B**), showed peak activity at concentration of ~0.1–1 nM, and a reduced activity at higher concentration.

Figures 12A and 12B show the correlation between the strength of binding to cells, and

10 the strength of NFκB activation (as determined as AUC for NFκB activation; **Figure 12A**) and the strength of T cell activation (**Figure 12B**) shown for trimeric, bispecific anti-OX40, anti-FAP antigen binding molecules. The binding strength is the MFI of at the highest concentration (see **Figure 5**) for each clone. The AUC of bioactivity is the AUC calculated for NFκB activation as in **Figure 8D**. The AUC of T cell bioactivity was calculated from dose response curves shown in

15 **Figure 11**. A negative correlation between binding strength to OX40 and agonism of bioactivity was observed. This may be explained by improved ability of molecules binding to OX40 with a low affinity (and high avidity) to dynamically recruit signaling OX40 receptor units. Low affinity binding may provide for a higher turn-over of receptor units by quick catch and release, whereas the high avidity still guarantees a clustering of several OX40 molecules at once and

20 hyper-crosslinking by FAP, for optimal receptor signaling.

Figures 13A to 13C show the binding of the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules to recombinant 4-1BB Fc (kih) receptor and human FAP protein, as assessed by surface plasmon resonance. **Figure 13A** shows binding of molecules comprising anti-4-1BB clone 25G7, **Figure 13B** shows binding of molecules comprising anti-4-1BB clone

25 12B3, and **Figure 13C** shows binding of molecules comprising anti-4-1BB clone 9B11.

Figures 14A to 14D show the binding of the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules; trimeric, bispecific isotype control DP47, anti-FAP antigen binding molecules; trimeric, monospecific anti-4-1BB antigen binding molecules; and isotype control DP47 huIgG P329G LALA molecules, to resting (naïve) and activated human CD4⁺ and CD8⁺

30 T cells. **Figure 14A** shows binding to naïve CD8⁺ T cells, **Figure 14B** shows binding to naïve CD4⁺ T cells, **Figure 14C** shows binding to activated CD8⁺ T cells, and **Figure 14D** shows binding to activated CD4⁺ T cells. Binding is shown as the median of fluorescence intensity (MFI) of PE conjugated anti-human IgG F(ab')₂-fragment-specific goat IgG F(ab')₂ fragment, which is used as secondary detection antibody. MFI was measured by flow cytometry and

35 baseline corrected by subtracting the MFI of the blank control. The x-axis shows the concentration of antigen binding molecules. All anti-4-1BB antigen binding molecules were

shown to bind to activated, 4-1BB expressing human CD4⁺ T cells and CD8⁺ T cells (**Figure 14C and 14D**), but did not display binding to naïve CD4⁺ and CD8⁺ T cells (**Figure 14A and 14B**).

Figure 15 shows the binding of the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules; trimeric, bispecific anti-4-1BB, isotype control DP47 antigen binding molecules; and monospecific, anti-4-1BB huIgG1 P329G LALA antigen binding molecules to FAP-expressing NIH/3T3-huFAP clone 39 cells. The trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules are efficiently targeted to FAP, whereas the molecules lacking anti-FAP scFv did not bind to FAP-expressing NIH/3T3-huFAP clone 39 cells.

Figures 16A and 16B show activation of NFκB by the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules, in the presence of crosslinking by FAP-positive cells. NF-κB-mediated luciferase activity was characterized by plotting the units of released light (URL), measured during 0.5 s, versus the concentration of the antigen binding molecule (in nM). URLs are emitted due to luciferase-mediated oxidation of luciferin to oxyluciferin (**Figure 16A**). The values were baseline-corrected by subtracting the URLs for a 'blank control' condition. **Figure 16B** shows that the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules (clone 25G7, filled diamonds and clone 12B3, filled squares) induced NFκB activation in human 4-1BB-positive HeLa reporter cells, but the control monospecific anti-4-1BB huIgG1 P329G LALA and trimeric monospecific anti-4-1BB antigen binding molecules lacking anti-FAP scFv did not trigger activation of NFκB.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as generally used in the art to which this invention belongs. For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa.

As used herein, the term "**antigen binding molecule**" refers in its broadest sense to a molecule that specifically binds an antigenic determinant. Examples of antigen binding molecules are antibodies, antibody fragments and scaffold antigen binding proteins.

As used herein, the term "**moiety capable of specific binding to a target cell antigen**" or "antigen binding domain capable of specific binding" refers to a polypeptide molecule that specifically binds to an antigenic determinant. In one aspect, the antigen binding moiety is able to activate signaling through its target cell antigen. In a particular aspect, the antigen binding

moiety is able to direct the entity to which it is attached to a target site, for example to a specific type of tumor cell or tumor stroma bearing the antigenic determinant. Moieties capable of specific binding to a target cell antigen include antibodies and fragments thereof as further defined herein. In one aspect, a "moiety capable of specific binding to a target cell antigen" is an antigen binding domain comprising an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH) capable of specific binding to a target cell antigen. In a particular aspect, the "moiety capable of specific binding to a costimulatory TNF receptor family member" may be a Fab fragment, a cross-Fab fragment or a scFv. In addition, moieties capable of specific binding to a target cell antigen include scaffold antigen binding proteins as further defined herein, e.g. binding domains which are based on designed repeat proteins or designed repeat domains (see e.g. WO 2002/020565).

In relation to an antibody or fragment thereof, the term "moiety capable of specific binding to a target cell antigen" refers to the part of the molecule that comprises the area which specifically binds to and is complementary to part or all of an antigen. A moiety capable of specific antigen binding may be provided, for example, by one or more antibody variable domains (also called antibody variable regions). Particularly, a moiety capable of specific antigen binding comprises an antibody light chain variable domain (VL) and an antibody heavy chain variable domain (VH). In some embodiments, the "moiety capable of specific binding to a target cell antigen" may be a scFv, a Fab fragment or a cross-Fab fragment.

The term "**moiety capable of specific binding to a costimulatory TNF receptor family member**" or "antigen binding domain capable of specific binding to a costimulatory TNF receptor family member" refers to a polypeptide molecule that specifically binds to a costimulatory TNF receptor family member. In one aspect, the antigen binding moiety is able to activate signaling through a costimulatory TNF receptor family member. Moieties capable of specific binding to a target cell antigen include antibodies and fragments thereof as further defined herein. In addition, moieties capable of specific binding to a costimulatory TNF receptor family member include scaffold antigen binding proteins as further defined herein, e.g. binding domains which are based on designed repeat proteins or designed repeat domains (see e.g. WO 2002/020565). Particularly, a moiety capable of specific binding to a costimulatory TNF receptor family member comprises an antibody light chain variable domain (VL) and an antibody heavy chain variable domain (VH). In a particular aspect, the "moiety capable of specific binding to a costimulatory TNF receptor family member" may be a Fab fragment, a cross-Fab fragment or a scFv.

The term "**antibody**" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies,

monospecific and multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

The term “**monoclonal antibody**” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g. containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen.

The term “**monospecific**” antibody as used herein denotes an antibody that has one or more binding sites each of which bind to the same epitope of the same antigen. The term “**bispecific**” means that the antigen binding molecule is able to specifically bind to at least two distinct antigenic determinants. A bispecific antigen binding molecule comprises at least two antigen binding sites, each of which is specific for a different antigenic determinant. In certain embodiments the bispecific antigen binding molecule is capable of simultaneously binding two antigenic determinants, particularly two antigenic determinants expressed on two distinct cells. For example, the trimeric antigen binding molecules of the present invention are bispecific, comprising a moiety capable of specific binding to a costimulatory TNF receptor family member and a moiety capable of specific binding to a target cell antigen.

The term “**valent**” as used within the current application denotes the presence of a specified number of binding sites in an antigen binding molecule. As such, the terms “bivalent”, “tetravalent”, and “hexavalent” denote the presence of two binding sites, four binding sites, and six binding sites, respectively, in an antigen binding molecule. Valency of an antigen binding molecule may also be expressed in relation to the number of binding sites for a given antigenic determinant. For example, the trimeric antigen binding molecules of the present invention are trivalent with respect to the target target cell antigen, and trivalent with respect to the costimulatory TNF receptor family member. Overall, the trimeric antigen binding molecules comprise six binding sites (3+3).

The terms “full length antibody”, “intact antibody”, and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure. “**Native antibodies**” refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG-class antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two light chains and two heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also

called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3), also called a heavy chain constant region. Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a light chain constant domain (CL), also called a light chain constant region. The heavy chain of an antibody may be assigned to one of five types, called α (IgA), δ (IgD), ϵ (IgE), γ (IgG), or μ (IgM), some of which may be further divided into subtypes, e.g. $\gamma 1$ (IgG1), $\gamma 2$ (IgG2), $\gamma 3$ (IgG3), $\gamma 4$ (IgG4), $\alpha 1$ (IgA1) and $\alpha 2$ (IgA2). The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

An "**antibody fragment**" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies, triabodies, tetrabodies, cross-Fab fragments; linear antibodies; single-chain antibody molecules (e.g. scFv); and single domain antibodies. For a review of certain antibody fragments, see Hudson et al., Nat Med 9, 129-134 (2003). For a review of scFv fragments, see e.g. Plückthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and F(ab')₂ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046. Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific, see, for example, EP 404,097; WO 1993/01161; Hudson et al., Nat Med 9, 129-134 (2003); and Hollinger et al., Proc Natl Acad Sci USA 90, 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., Nat Med 9, 129-134 (2003). Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; see e.g. U.S. Patent No. 6,248,516 B1). Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. E. coli or phage), as described herein.

Papain digestion of intact antibodies produces two identical antigen-binding fragments, called "Fab" fragments containing each the heavy- and light-chain variable domains and also the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. As used herein, Thus, the term "**Fab fragment**" refers to an antibody fragment comprising a light chain fragment comprising a light chain variable domain (VL) and a constant domain of a light chain (CL), and a heavy chain variable domain (VH) and a first constant domain (CH1) of a heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteins from the

antibody hinge region. Fab'-SH are Fab' fragments in which the cysteine residue(s) of the constant domains bear a free thiol group. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-combining sites (two Fab fragments) and a part of the Fc region.

The term “**cross-Fab fragment**” or “xFab fragment” or “crossover Fab fragment” refers to a Fab fragment, wherein either the variable regions or the constant regions of the heavy and light chain are exchanged. Two different chain compositions of a crossover Fab molecule are possible and comprised in the bispecific antibodies of the invention: On the one hand, the variable regions of the Fab heavy and light chain are exchanged, i.e. the crossover Fab molecule comprises a peptide chain composed of the light chain variable region (VL) and the heavy chain constant region (CH1), and a peptide chain composed of the heavy chain variable region (VH) and the light chain constant region (CL). This crossover Fab molecule is also referred to as CrossFab_(VLVH). On the other hand, when the constant regions of the Fab heavy and light chain are exchanged, the crossover Fab molecule comprises a peptide chain composed of the heavy chain variable region (VH) and the light chain constant region (CL), and a peptide chain composed of the light chain variable region (VL) and the heavy chain constant region (CH1). This crossover Fab molecule is also referred to as CrossFab_(CLCH1).

A “single chain Fab fragment” or “**scFab**” is a polypeptide consisting of an antibody heavy chain variable domain (VH), an antibody constant domain 1 (CH1), an antibody light chain variable domain (VL), an antibody light chain constant domain (CL) and a linker, wherein said antibody domains and said linker have one of the following orders in N-terminal to C-terminal direction: a) VH-CH1-linker-VL-CL, b) VL-CL-linker-VH-CH1, c) VH-CL-linker-VL-CH1 or d) VL-CH1-linker-VH-CL; and wherein said linker is a polypeptide of at least 30 amino acids, preferably between 32 and 50 amino acids. Said single chain Fab fragments are stabilized via the natural disulfide bond between the CL domain and the CH1 domain. In addition, these single chain Fab molecules might be further stabilized by generation of interchain disulfide bonds via insertion of cysteine residues (e.g. position 44 in the variable heavy chain and position 100 in the variable light chain according to Kabat numbering).

A “crossover single chain Fab fragment” or “**x-scFab**” is a polypeptide consisting of an antibody heavy chain variable domain (VH), an antibody constant domain 1 (CH1), an antibody light chain variable domain (VL), an antibody light chain constant domain (CL) and a linker, wherein said antibody domains and said linker have one of the following orders in N-terminal to C-terminal direction: a) VH-CL-linker-VL-CH1 and b) VL-CH1-linker-VH-CL; wherein VH and VL form together an antigen-binding site which binds specifically to an antigen and wherein said linker is a polypeptide of at least 30 amino acids. In addition, these x-scFab molecules might be further stabilized by generation of interchain disulfide bonds via insertion of

cysteine residues (e.g. position 44 in the variable heavy chain and position 100 in the variable light chain according to Kabat numbering).

A “**single-chain variable fragment (scFv)**” is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of an antibody, connected with a short linker peptide of ten 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 V_H with the C-terminus of the V_L , or *vice versa*. This protein retains the specificity of the original antibody, despite removal of the constant regions and the introduction of the linker. scFv antibodies are, e.g. described in Houston, J.S., Methods in Enzymol. 203 (1991) 46-96). In addition, antibody fragments comprise single chain polypeptides having the characteristics of a V_H domain, namely being able to assemble together with a V_L domain, or of a V_L domain, namely being able to assemble together with a V_H domain to a functional antigen binding site and thereby providing the antigen binding property of full length antibodies.

“**Scaffold antigen binding proteins**” are known in the art, for example, fibronectin and designed ankyrin repeat proteins (DARPin) have been used as alternative scaffolds for antigen-binding domains, see, e.g., Gebauer and Skerra, Engineered protein scaffolds as next-generation antibody therapeutics. Curr Opin Chem Biol 13:245-255 (2009) and Stumpp et al., Darpins: A new generation of protein therapeutics. Drug Discovery Today 13: 695-701 (2008). In one aspect of the invention, a scaffold antigen binding protein is selected from the group consisting of CTLA-4 (Evibody), Lipocalins (Anticalin), a Protein A-derived molecule such as Z-domain of Protein A (Affibody), an A-domain (Avimer/Maxibody), a serum transferrin (*trans*-body); a designed ankyrin repeat protein (DARPin), a variable domain of antibody light chain or heavy chain (single-domain antibody, sdAb), a variable domain of antibody heavy chain (nanobody, a V_H), V_{NAR} fragments, a fibronectin (AdNectin), a C-type lectin domain (Tetranectin); a variable domain of a new antigen receptor beta-lactamase (V_{NAR} fragments), a human gamma-crystallin or ubiquitin (Affilin molecules); a kunitz type domain of human protease inhibitors, microbodies such as the proteins from the knottin family, peptide aptamers and fibronectin (adnectin).

CTLA-4 (Cytotoxic T Lymphocyte-associated Antigen 4) is a CD28-family receptor expressed on mainly CD4⁺ T-cells. Its extracellular domain has a variable domain- like Ig fold. Loops corresponding to CDRs of antibodies can be substituted with heterologous sequence to confer different binding properties. CTLA-4 molecules engineered to have different binding specificities are also known as Evibodies (e.g. US7166697B1). Evibodies are around the same size as the isolated variable region of an antibody (e.g. a domain antibody). For further details see Journal of Immunological Methods 248 (1-2), 31-45 (2001).

Lipocalins are a family of extracellular proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids. They have a rigid beta-sheet secondary structure with a number of loops at the open end of the conical structure which can be engineered to bind to different target antigens. Anticalins are between 160-180 amino acids in size, and are
5 derived from lipocalins. For further details see Biochim Biophys Acta 1482: 337-350 (2000), US7250297B1 and US20070224633.

An affibody is a scaffold derived from Protein A of *Staphylococcus aureus* which can be engineered to bind to antigen. The domain consists of a three-helical bundle of approximately 58 amino acids. Libraries have been generated by randomization of surface residues. For further
10 details see Protein Eng. Des. Sel. 17, 455-462 (2004) and EP 1641818A1.

Avimers are multidomain proteins derived from the A-domain scaffold family. The native domains of approximately 35 amino acids adopt a defined disulfide bonded structure. Diversity is generated by shuffling of the natural variation exhibited by the family of A-domains. For further details see Nature Biotechnology 23(12), 1556 - 1561 (2005) and Expert Opinion on
15 Investigational Drugs 16(6), 909-917 (June 2007).

A transferrin is a monomeric serum transport glycoprotein. Transferrins can be engineered to bind different target antigens by insertion of peptide sequences in a permissive surface loop. Examples of engineered transferrin scaffolds include the Trans-body. For further details see J. Biol. Chem 274, 24066-24073 (1999).

Designed Ankyrin Repeat Proteins (DARPin)s are derived from Ankyrin which is a family of proteins that mediate attachment of integral membrane proteins to the cytoskeleton. A single ankyrin repeat is a 33 residue motif consisting of two alpha-helices and a beta-turn. They can be engineered to bind different target antigens by randomizing residues in the first alpha-helix and a beta-turn of each repeat. Their binding interface can be increased by increasing the number of
20 modules (a method of affinity maturation). For further details see J. Mol. Biol. 332, 489-503 (2003), PNAS 100(4), 1700-1705 (2003) and J. Mol. Biol. 369, 1015-1028 (2007) and US20040132028A1.

A single-domain antibody is an antibody fragment consisting of a single monomeric variable antibody domain. The first single domains were derived from the variable domain of the
30 antibody heavy chain from camelids (nanobodies or V_HH fragments). Furthermore, the term single-domain antibody includes an autonomous human heavy chain variable domain (aVH) or V_{NAR} fragments derived from sharks.

Fibronectin is a scaffold which can be engineered to bind to antigen. Adnectins consists of a backbone of the natural amino acid sequence of the 10th domain of the 15 repeating units of

human fibronectin type III (FN3). Three loops at one end of the .beta.-sandwich can be engineered to enable an Adnectin to specifically recognize a therapeutic target of interest. For further details see Protein Eng. Des. Sel. 18, 435- 444 (2005), US20080139791, WO2005056764 and US6818418B1.

5 Peptide aptamers are combinatorial recognition molecules that consist of a constant scaffold protein, typically thioredoxin (TrxA) which contains a constrained variable peptide loop inserted at the active site. For further details see Expert Opin. Biol. Ther. 5, 783-797 (2005).

10 Microbodies are derived from naturally occurring microproteins of 25-50 amino acids in length which contain 3-4 cysteine bridges - examples of microproteins include KalataBI and conotoxin and knottins. The microproteins have a loop which can be engineered to include upto 25 amino acids without affecting the overall fold of the microprotein. For further details of engineered knottin domains, see WO2008098796.

15 An “**antigen binding molecule that binds to the same epitope**” as a reference molecule refers to an antigen binding molecule that blocks binding of the reference molecule to its antigen in a competition assay by 50% or more, and conversely, the reference molecule blocks binding of the antigen binding molecule to its antigen in a competition assay by 50% or more.

20 The term “**antigen binding domain**” refers to the part of an antigen binding molecule that comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antigen binding molecule may only bind to a particular part of the antigen, which part is termed an epitope. An antigen binding domain may be provided by, for example, one or more variable domains (also called variable regions). Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

25 As used herein, the term “**antigenic determinant**” is synonymous with “antigen” and “epitope,” and refers to a site (e.g. a contiguous stretch of amino acids or a conformational configuration made up of different regions of non-contiguous amino acids) on a polypeptide macromolecule to which an antigen binding moiety binds, forming an antigen binding moiety-antigen complex. Useful antigenic determinants can be found, for example, on the surfaces of tumor cells, on the surfaces of virus-infected cells, on the surfaces of other diseased cells, on the surface of immune cells, free in blood serum, and/or in the extracellular matrix (ECM). The proteins useful as antigens herein can be any native form the proteins from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g. mice and rats), unless otherwise indicated. In a particular embodiment the antigen is a human protein. Where reference is made to a specific protein herein, the term encompasses the “full-length”, unprocessed protein

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as well as any form of the protein that results from processing in the cell. The term also encompasses naturally occurring variants of the protein, e.g. splice variants or allelic variants.

By "**specific binding**" is meant that the binding is selective for the antigen and can be discriminated from unwanted or non-specific interactions. The ability of an antigen binding molecule to bind to a specific antigen can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. Surface Plasmon Resonance (SPR) technique (analyzed on a BIAcore instrument) (Liljeblad et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). In one embodiment, the extent of binding of an antigen binding molecule to an unrelated protein is less than about 10% of the binding of the antigen binding molecule to the antigen as measured, e.g. by SPR. In certain embodiments, an molecule that binds to the antigen has a dissociation constant (K_d) of $\leq 1 \mu\text{M}$, $\leq 100 \text{ nM}$, $\leq 10 \text{ nM}$, $\leq 1 \text{ nM}$, $\leq 0.1 \text{ nM}$, $\leq 0.01 \text{ nM}$, or $\leq 0.001 \text{ nM}$ (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M , e.g. from 10^{-9} M to 10^{-13} M).

"**Affinity**" or "binding affinity" refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g. an antibody) and its binding partner (e.g. an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g. antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_d), which is the ratio of dissociation and association rate constants (k_{off} and k_{on} , respectively). Thus, equivalent affinities may comprise different rate constants, as long as the ratio of the rate constants remains the same. Affinity can be measured by common methods known in the art, including those described herein. A particular method for measuring affinity is Surface Plasmon Resonance (SPR).

A "**target cell antigen**" as used herein refers to an antigenic determinant presented on the surface of a target cell, for example a cell in a tumor such as a cancer cell or a cell of the tumor stroma. In certain embodiments, the target cell antigen is an antigen on the surface of a tumor cell. In one embodiment, target cell antigen is selected from the group consisting of Fibroblast Activation Protein (FAP), Carcinoembryonic Antigen (CEA), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), CD19, CD20 and CD33. In particular, the target cell antigen is Fibroblast Activation Protein (FAP).

The term "**Fibroblast activation protein (FAP)**", also known as Prolyl endopeptidase FAP or Seprase (EC 3.4.21), refers to any native FAP from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed FAP as well as any form of FAP that results from processing in the cell. The term

also encompasses naturally occurring variants of FAP, e.g., splice variants or allelic variants. In one embodiment, the antigen binding molecule of the invention is capable of specific binding to human, mouse and/or cynomolgus FAP. The amino acid sequence of human FAP is shown in UniProt (www.uniprot.org) accession no. Q12884 (version 149, SEQ ID NO:86), or NCBI
5 (www.ncbi.nlm.nih.gov/) RefSeq NP_004451.2. The extracellular domain (ECD) of human FAP extends from amino acid position 26 to 760. The amino acid and nucleotide sequences of a His-tagged human FAP ECD is shown in SEQ ID NOs 87 and 88, respectively. The amino acid sequence of mouse FAP is shown in UniProt accession no. P97321 (version 126, SEQ ID NO:89), or NCBI RefSeq NP_032012.1. The extracellular domain (ECD) of mouse FAP extends
10 from amino acid position 26 to 761. SEQ ID NOs 90 and 91 show the amino acid and nucleotide sequences, respectively, of a His-tagged mouse FAP ECD. SEQ ID NOs 92 and 93 show the amino acid and nucleotide sequences, respectively, of a His-tagged cynomolgus FAP ECD. Preferably, an anti-FAP binding molecule of the invention binds to the extracellular domain of FAP. Exemplary anti-FAP binding molecules are described in International Patent Application
15 No. WO 2012/020006 A2.

The term “**Carcinoembryonic antigen (CEA)**”, also known as Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), refers to any native CEA from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The amino
20 acid sequence of human CEA is shown in UniProt accession no. P06731 (version 151, SEQ ID NO:94). CEA has long been identified as a tumor-associated antigen (Gold and Freedman, J Exp Med., 121:439-462, 1965; Berinstein N. L., J Clin Oncol., 20:2197-2207, 2002). Originally classified as a protein expressed only in fetal tissue, CEA has now been identified in several normal adult tissues. These tissues are primarily epithelial in origin, including cells of the
25 gastrointestinal, respiratory, and urogenital tracts, and cells of colon, cervix, sweat glands, and prostate (Nap et al., Tumour Biol., 9(2-3):145-53, 1988; Nap et al., Cancer Res., 52(8):2329-23339, 1992). Tumors of epithelial origin, as well as their metastases, contain CEA as a tumor associated antigen. While the presence of CEA itself does not indicate transformation to a cancerous cell, the distribution of CEA is indicative. In normal tissue, CEA is generally
30 expressed on the apical surface of the cell (Hammarström S., Semin Cancer Biol. 9(2):67-81 (1999)), making it inaccessible to antibody in the blood stream. In contrast to normal tissue, CEA tends to be expressed over the entire surface of cancerous cells (Hammarström S., Semin Cancer Biol. 9(2):67-81 (1999)). This change of expression pattern makes CEA accessible to antibody binding in cancerous cells. In addition, CEA expression increases in cancerous cells.
35 Furthermore, increased CEA expression promotes increased intercellular adhesions, which may lead to metastasis (Marshall J., Semin Oncol., 30(a Suppl. 8):30-6, 2003). The prevalence of CEA expression in various tumor entities is generally very high. In concordance with published

data, own analyses performed in tissue samples confirmed its high prevalence, with approximately 95% in colorectal carcinoma (CRC), 90% in pancreatic cancer, 80% in gastric cancer, 60% in non-small cell lung cancer (NSCLC, where it is co-expressed with HER3), and 40% in breast cancer; low expression was found in small cell lung cancer and glioblastoma.

5 CEA is readily cleaved from the cell surface and shed into the blood stream from tumors, either directly or via the lymphatics. Because of this property, the level of serum CEA has been used as a clinical marker for diagnosis of cancers and screening for recurrence of cancers, particularly colorectal cancer (Goldenberg D M., The International Journal of Biological Markers, 7:183-188, 1992; Chau I., et al., J Clin Oncol., 22:1420-1429, 2004; Flamini et al., Clin
10 Cancer Res; 12(23):6985-6988, 2006).

The term “**Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP)**”, also known as Chondroitin Sulfate Proteoglycan 4 (CSPG4) refers to any native MCSP from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The amino
15 acid sequence of human MCSP is shown in UniProt accession no. Q6UVK1 (version 103, SEQ ID NO:95). The term “**Epidermal Growth Factor Receptor (EGFR)**”, also named Proto-oncogene c-ErbB-1 or Receptor tyrosine-protein kinase erbB-1, refers to any native EGFR from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The
20 amino acid sequence of human EGFR is shown in UniProt accession no. P00533 (version 211, SEQ ID NO:96).

The term “**CD19**” refers to B-lymphocyte antigen CD19, also known as B-lymphocyte surface antigen B4 or T-cell surface antigen Leu-12 and includes any native CD19 from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g.
25 cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The amino acid sequence of human CD19 is shown in Uniprot accession no. P15391 (version 160, SEQ ID NO:97). The term encompasses “full-length” unprocessed human CD19 as well as any form of human CD19 that results from processing in the cell as long as the antibody as reported herein binds thereto. CD19 is a structurally distinct cell surface receptor expressed on the surface of
30 human B cells, including, but not limited to, pre-B cells, B cells in early development {i.e., immature B cells}, mature B cells through terminal differentiation into plasma cells, and malignant B cells. CD19 is expressed by most pre-B acute lymphoblastic leukemias (ALL), non-Hodgkin's lymphomas, B cell chronic lymphocytic leukemias (CLL), pro-lymphocytic leukemias, hairy cell leukemias, common acute lymphocytic leukemias, and some Null-acute lymphoblastic
35 leukemias. The expression of CD19 on plasma cells further suggests it may be expressed on differentiated B cell tumors such as multiple myeloma. Therefore, the CD19 antigen is a target

for immunotherapy in the treatment of non-Hodgkin's lymphoma, chronic lymphocytic leukemia and/or acute lymphoblastic leukemia.

“**CD20**” refers to B-lymphocyte antigen CD20, also known as membrane-spanning 4-domains subfamily A member 1 (MS4A1), B-lymphocyte surface antigen B1 or Leukocyte surface antigen Leu-16, and includes any native CD20 from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The amino acid sequence of human CD20 is shown in Uniprot accession no. P11836 (version 149, SEQ ID NO:98). “**CD33**” refers to Myeloid cell surface antigen CD33, also known as SIGLEC3 or gp67, and includes any native CD33 from any vertebrate source, including mammals such as primates (e.g. humans) non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The amino acid sequence of human CD33 is shown in Uniprot accession no. P20138 (version 157, SEQ ID NO:99).

The term “**variable region**” or “variable domain” refers to the domain of an antibody heavy or light chain that is involved in binding the antigen binding molecule to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). See, e.g., Kindt et al., Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007). A single VH or VL may be sufficient to confer antigen-binding specificity.

The term “**hypervariable region**” or “HVR,” as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops (“hypervariable loops”). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the “complementarity determining regions” (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition. Exemplary hypervariable loops occur at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3). (Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987).) Exemplary CDRs (CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3) occur at amino acid residues 24-34 of L1, 50-56 of L2, 89-97 of L3, 31-35B of H1, 50-65 of H2, and 95-102 of H3. (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991).) Hypervariable regions (HVRs) are also referred to as complementarity determining regions (CDRs), and these terms are used herein interchangeably in reference to portions of the variable region that form the antigen binding regions. This particular region has been described by Kabat et al., U.S. Dept. of Health and Human Services, “Sequences of Proteins of Immunological

Interest" (1983) and by Chothia *et al.*, *J. Mol. Biol.* 196:901-917 (1987), where the definitions include overlapping or subsets of amino acid residues when compared against each other.

Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in **Table A** as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody

Table A. CDR Definitions¹

CDR	Kabat	Chothia	AbM²
V _H CDR1	31-35	26-32	26-35
V _H CDR2	50-65	52-58	50-58
V _H CDR3	95-102	95-102	95-102
V _L CDR1	24-34	26-32	24-34
V _L CDR2	50-56	50-52	50-56
V _L CDR3	89-97	91-96	89-97

¹ Numbering of all CDR definitions in **Table A** is according to the numbering conventions set forth by Kabat *et al.* (see below).

² "AbM" with a lowercase "b" as used in **Table A** refers to the CDRs as defined by Oxford Molecular's "AbM" antibody modeling software.

Kabat *et al.* also defined a numbering system for variable region sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable region sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat *et al.*, U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983). Unless otherwise specified, references to the numbering of specific amino acid residue positions in an antibody variable region are according to the Kabat numbering system.

With the exception of CDR1 in V_H, CDRs generally comprise the amino acid residues that form the hypervariable loops. CDRs also comprise "specificity determining residues," or "SDRs," which are residues that contact antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Exemplary a-CDRs (a-CDR-L1, a-CDR-L2, a-CDR-L3, a-CDR-H1, a-CDR-H2, and a-CDR-H3) occur at amino acid residues 31-34 of L1, 50-55 of L2, 89-96 of L3, 31-35B of H1, 50-58 of H2, and 95-102 of H3. (See Almagro and Fransson, *Front.*

Biosci. 13:1619-1633 (2008).) Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*.

As used herein, the term “**affinity matured**” in the context of antigen binding molecules (e.g., antibodies) refers to an antigen binding molecule that is derived from a reference antigen binding molecule, e.g., by mutation, binds to the same antigen, preferably binds to the same epitope, as the reference antibody; and has a higher affinity for the antigen than that of the reference antigen binding molecule. Affinity maturation generally involves modification of one or more amino acid residues in one or more CDRs of the antigen binding molecule. Typically, the affinity matured antigen binding molecule binds to the same epitope as the initial reference antigen binding molecule.

“**Framework**” or “FR” refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

An “**acceptor human framework**” for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework “derived from” a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

The term “**chimeric**” antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

The “**class**” of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ respectively.

A “**humanized**” antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a

humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region
5 derived from a human antibody. A “**humanized form**” of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization. Other forms of “humanized antibodies” encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to C1q binding and/or Fc receptor
10 (FcR) binding.

A “**human**” antibody is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-
15 human antigen-binding residues.

The term “Fc domain” or “**Fc region**” herein is used to define a C-terminal region of an antibody heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. An IgG Fc region comprises an IgG CH2 and an IgG CH3 domain. The “CH2 domain” of a human IgG Fc region usually extends from an
20 amino acid residue at about position 231 to an amino acid residue at about position 340. In one embodiment, a carbohydrate chain is attached to the CH2 domain. The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain. The “CH3 domain” comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (i.e. from an amino acid residue at about position 341 to an amino acid residue at about position 447 of an IgG). The CH3 region
25 herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced “protuberance” (“knob”) in one chain thereof and a corresponding introduced “cavity” (“hole”) in the other chain thereof; see US Patent No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to promote heterodimerization of two non-identical antibody heavy chains as herein described. In one embodiment, a human IgG
30 heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service,
35 National Institutes of Health, Bethesda, MD, 1991.

The “**knob-into-hole**” technology is described e.g. in US 5,731,168; US 7,695,936; Ridgway et al., Prot Eng 9, 617-621 (1996) and Carter, J Immunol Meth 248, 7-15 (2001). Generally, the method involves introducing a protuberance (“knob”) at the interface of a first polypeptide and a corresponding cavity (“hole”) in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). The protuberance and cavity can be made by altering the nucleic acid encoding the polypeptides, e.g. by site-specific mutagenesis, or by peptide synthesis. In a specific embodiment a knob modification comprises the amino acid substitution T366W in one of the two subunits of the Fc domain, and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V in the other one of the two subunits of the Fc domain. In a further specific embodiment, the subunit of the Fc domain comprising the knob modification additionally comprises the amino acid substitution S354C, and the subunit of the Fc domain comprising the hole modification additionally comprises the amino acid substitution Y349C. Introduction of these two cysteine residues results in the formation of a disulfide bridge between the two subunits of the Fc region, thus further stabilizing the dimer (Carter, J Immunol Methods 248, 7-15 (2001)).

A “region equivalent to the Fc region of an immunoglobulin” is intended to include naturally occurring allelic variants of the Fc region of an immunoglobulin as well as variants having alterations which produce substitutions, additions, or deletions but which do not decrease substantially the ability of the immunoglobulin to mediate effector functions (such as antibody-dependent cellular cytotoxicity). For example, one or more amino acids can be deleted from the N-terminus or C-terminus of the Fc region of an immunoglobulin without substantial loss of biological function. Such variants can be selected according to general rules known in the art so as to have minimal effect on activity (see, e.g., Bowie, J. U. et al., Science 247:1306-10 (1990)).

The term “**effector functions**” refers to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC), Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), cytokine secretion, immune complex-mediated antigen uptake by antigen presenting cells, down regulation of cell surface receptors (e.g. B cell receptor), and B cell activation.

Fc receptor binding dependent effector functions can be mediated by the interaction of the Fc-region of an antibody with Fc receptors (FcRs), which are specialized cell surface receptors on hematopoietic cells. Fc receptors belong to the immunoglobulin superfamily, and have been shown to mediate both the removal of antibody-coated pathogens by phagocytosis of immune complexes, and the lysis of erythrocytes and various other cellular targets (e.g. tumor cells) coated with the corresponding antibody, via antibody dependent cell mediated cytotoxicity (ADCC) (see e.g. Van de Winkel, J.G. anderson, C.L., *J. Leukoc. Biol.* 49 (1991) 511-524). FcRs are defined by their specificity for immunoglobulin isotypes: Fc receptors for IgG antibodies are referred to as Fc γ R. Fc receptor binding is described e.g. in Ravetch, J.V. and Kinet, J.P., *Annu. Rev. Immunol.* 9 (1991) 457-492; Capel, P.J., et al., *Immunomethods* 4 (1994) 25-34; de Haas, M., et al., *J. Lab. Clin. Med.* 126 (1995) 330-341; and Gessner, J.E., et al., *Ann. Hematol.* 76 (1998) 231-248.

Cross-linking of receptors for the Fc-region of IgG antibodies (Fc γ R) triggers a wide variety of effector functions including phagocytosis, antibody-dependent cellular cytotoxicity, and release of inflammatory mediators, as well as immune complex clearance and regulation of antibody production. In humans, three classes of Fc γ R have been characterized, which are:

- Fc γ RI (CD64) binds monomeric IgG with high affinity and is expressed on macrophages, monocytes, neutrophils and eosinophils. Modification in the Fc-region IgG at least at one of the amino acid residues E233-G236, P238, D265, N297, A327 and P329 (numbering according to EU index of Kabat) reduce binding to Fc γ RI. IgG2 residues at positions 233-236, substituted into IgG1 and IgG4, reduced binding to Fc γ RI by 10³-fold and eliminated the human monocyte response to antibody-sensitized red blood cells (Armour, K.L., et al., *Eur. J. Immunol.* 29 (1999) 2613-2624).

- Fc γ RII (CD32) binds complexed IgG with medium to low affinity and is widely expressed. This receptor can be divided into two sub-types, Fc γ RIIA and Fc γ RIIB. Fc γ RIIA is found on many cells involved in killing (e.g. macrophages, monocytes, neutrophils) and seems able to activate the killing process. Fc γ RIIB seems to play a role in inhibitory processes and is found on B cells, macrophages and on mast cells and eosinophils. On B-cells it seems to function to suppress further immunoglobulin production and isotype switching to, for example, the IgE class. On macrophages, Fc γ RIIB acts to inhibit phagocytosis as mediated through Fc γ RIIA. On eosinophils and mast cells the B-form may help to suppress activation of these cells through IgE binding to its separate receptor. Reduced binding for Fc γ RIIA is found e.g. for antibodies comprising an IgG Fc-region with mutations at least at one of the amino acid residues E233-G236, P238, D265, N297, A327, P329, D270, Q295, A327, R292, and K414 (numbering according to EU index of Kabat).

- Fc γ RIII (CD16) binds IgG with medium to low affinity and exists as two types. Fc γ RIIIA is found on NK cells, macrophages, eosinophils and some monocytes and T cells and mediates

ADCC. Fc γ RIIIB is highly expressed on neutrophils. Reduced binding to Fc γ RIIA is found e.g. for antibodies comprising an IgG Fc-region with mutation at least at one of the amino acid residues E233-G236, P238, D265, N297, A327, P329, D270, Q295, A327, S239, E269, E293, Y296, V303, A327, K338 and D376 (numbering according to EU index of Kabat).

5 Mapping of the binding sites on human IgG1 for Fc receptors, the above mentioned mutation sites and methods for measuring binding to Fc γ RI and Fc γ RIIA are described in Shields, R.L., et al. J. Biol. Chem. 276 (2001) 6591-6604.

The term “**ADCC**” or “antibody-dependent cellular cytotoxicity” is a function mediated by Fc receptor binding and refers to lysis of target cells by an antibody as reported herein in the presence of effector cells. The capacity of the antibody to induce the initial steps mediating ADCC is investigated by measuring their binding to Fc γ receptors expressing cells, such as cells, recombinantly expressing Fc γ RI and/or Fc γ RIIA or NK cells (expressing essentially Fc γ RIIA). In particular, binding to Fc γ R on NK cells is measured.

15 An “**activating Fc receptor**” is an Fc receptor that following engagement by an Fc region of an antibody elicits signaling events that stimulate the receptor-bearing cell to perform effector functions. Activating Fc receptors include Fc γ RIIIa (CD16a), Fc γ RI (CD64), Fc γ RIIa (CD32), and Fc α RI (CD89). A particular activating Fc receptor is human Fc γ RIIIa (see UniProt accession no. P08637, version 141).

20 The “Tumor Necrosis factor receptor superfamily” or “**TNF receptor superfamily**” currently consists of 27 receptors. It is a group of cytokine receptors characterized by the ability to bind tumor necrosis factors (TNFs) via an extracellular cysteine-rich domain (CRD). These pseudorepeats are defined by intrachain disulphides generated by highly conserved cysteine residues within the receptor chains. With the exception of nerve growth factor (NGF), all TNFs are homologous to the archetypal TNF-alpha. In their active form, the majority of TNF receptors form trimeric complexes in the plasma membrane. Accordingly, most TNF receptors contain transmembrane domains (TMDs). Several of these receptors also contain intracellular death domains (DDs) that recruit caspase-interacting proteins following ligand binding to initiate the extrinsic pathway of caspase activation. Other TNF superfamily receptors that lack death domains bind TNF receptor-associated factors and activate intracellular signaling pathways that can lead to proliferation or differentiation. These receptors can also initiate apoptosis, but they do so via indirect mechanisms. In addition to regulating apoptosis, several TNF superfamily receptors are involved in regulating immune cell functions such as B cell homeostasis and activation, natural killer cell activation, and T cell co-stimulation. Several others regulate cell type-specific responses such as hair follicle development and osteoclast development. Members of the TNF receptor superfamily include the following: Tumor necrosis factor receptor 1 (1A) (TNFRSF1A, CD120a), Tumor necrosis factor receptor 2 (1B) (TNFRSF1B, CD120b),

Lymphotoxin beta receptor (LTBR, CD18), OX40 (TNFRSF4, CD134), CD40 (Bp50), Fas receptor (Apo-1, CD95, FAS), Decoy receptor 3 (TR6, M68, TNFRSF6B), CD27 (S152, Tp55), CD30 (Ki-1, TNFRSF8), 4-1BB (CD137, TNFRSF9), DR4 (TRAILR1, Apo-2, CD261, TNFRSF10A), DR5 (TRAILR2, CD262, TNFRSF10B), Decoy Receptor 1 (TRAILR3, CD263, TNFRSF10C), Decoy Receptor 2 (TRAILR4, CD264, TNFRSF10D), RANK (CD265, TNFRSF11A), Osteoprotegerin (OCIF, TR1, TNFRSF11B), TWEAK receptor (Fn14, CD266, TNFRSF12A), TACI (CD267, TNFRSF13B), BAFF receptor (CD268, TNFRSF13C), Herpesvirus entry mediator (HVEM, TR2, CD270, TNFRSF14), Nerve growth factor receptor (p75NTR, CD271, NGFR), B-cell maturation antigen (CD269, TNFRSF17), Glucocorticoid-induced TNFR-related (GITR, AITR, CD357, TNFRSF18), TROY (TNFRSF19), DR6 (CD358, TNFRSF21), DR3 (Apo-3, TRAMP, WS-1, TNFRSF25) and Ectodysplasin A2 receptor (XEDAR, EDA2R).

Several members of the tumor necrosis factor receptor (TNFR) family function after initial T cell activation to sustain T cell responses. The term “**costimulatory TNF receptor family member**” or “costimulatory TNF family receptor” refers to a subgroup of TNF receptor family members, which are able to costimulate proliferation and cytokine production of T-cells. The term refers to any native TNF family receptor from any vertebrate source, including mammals such as primates (e.g. humans), non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. In specific embodiments of the invention, costimulatory TNF receptor family members are selected from the group consisting of OX40 (CD134), 4-1BB (CD137), CD27, HVEM (CD270), CD30, and GITR, all of which can have costimulatory effects on T cells. More particularly, the costimulatory TNF receptor family member is selected from the group consisting of OX40 and 4-1BB.

Further information, in particular sequences, of the TNF receptor family members may be obtained from publically accessible databases such as Uniprot (www.uniprot.org). For instance, the human costimulatory TNF receptors have the following amino acid sequences: human OX40 (UniProt accession no. P43489, SEQ ID NO:100), human 4-1BB (UniProt accession no. Q07011, SEQ ID NO:101), human CD27 (UniProt accession no. P26842, SEQ ID NO:102), human HVEM (UniProt accession no. Q92956, SEQ ID NO:103), human CD30 (UniProt accession no. P28908, SEQ ID NO:104), and human GITR (UniProt accession no. Q9Y5U5, SEQ ID NO:105).

The term “**OX40**”, as used herein, refers to any native OX40 from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses “full-length,” unprocessed OX40 as well as any form of OX40 that results from processing in the cell. The term also encompasses naturally occurring variants of OX40, e.g., splice variants or allelic variants. The amino acid sequence of an

exemplary human OX40 is shown in SEQ ID NO: 100 (Uniprot P43489, version 112) and the amino acid sequence of an exemplary murine OX40 is shown in SEQ ID NO: 106 (Uniprot P47741, version 101).

The terms “**anti-OX40 antibody**”, “anti-OX40”, “OX40 antibody and “an antibody that specifically binds to OX40” refer to an antibody that is capable of binding OX40 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting OX40. In one embodiment, the extent of binding of an anti-OX40 antibody to an unrelated, non-OX40 protein is less than about 10% of the binding of the antibody to OX40 as measured, e.g., by a radioimmunoassay (RIA) or flow cytometry (FACS). In certain embodiments, an antibody that binds to OX40 has a dissociation constant (K_D) of $\leq 1\mu\text{M}$, $\leq 100\text{ nM}$, $\leq 10\text{ nM}$, $\leq 1\text{ nM}$, $\leq 0.1\text{ nM}$, $\leq 0.01\text{ nM}$, or $\leq 0.001\text{ nM}$ (e.g. 10^{-6} M or less, e.g. from 10^{-68} M to 10^{-13} M , e.g., from 10^{-8} M to 10^{-10} M).

The term “**4-1BB**”, as used herein, refers to any native 4-1BB from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses “full-length,” unprocessed 4-1BB as well as any form of 4-1BB that results from processing in the cell. The term also encompasses naturally occurring variants of 4-1BB, e.g., splice variants or allelic variants. The amino acid sequence of an exemplary human 4-1BB is shown in SEQ ID NO: 101 (Uniprot accession no. Q07011), the amino acid sequence of an exemplary murine 4-1BB is shown in SEQ ID NO: 107 (Uniprot accession no. P20334) and the amino acid sequence of an exemplary cynomolgous 4-1BB (from *Macaca mulatta*) is shown in SEQ ID NO:108 (Uniprot accession no. F6W5G6).

The terms “**anti-4-1BB antibody**”, “anti-4-1BB”, “4-1BB antibody and “an antibody that specifically binds to 4-1BB” refer to an antibody that is capable of binding 4-1BB with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting 4-1BB. In one embodiment, the extent of binding of an anti-4-1BB antibody to an unrelated, non-4-1BB protein is less than about 10% of the binding of the antibody to 4-1BB as measured, e.g., by a radioimmunoassay (RIA) or flow cytometry (FACS). In certain embodiments, an antibody that binds to 4-1BB has a dissociation constant (K_D) of $\leq 1\mu\text{M}$, $\leq 100\text{ nM}$, $\leq 10\text{ nM}$, $\leq 1\text{ nM}$, $\leq 0.1\text{ nM}$, $\leq 0.01\text{ nM}$, or $\leq 0.001\text{ nM}$ (e.g. 10^{-6} M or less, e.g. from 10^{-68} M to 10^{-13} M , e.g., from 10^{-8} M to 10^{-10} M).

The term “**peptide linker**” refers to a peptide comprising one or more amino acids, typically about 2 to 20 amino acids. Peptide linkers are known in the art or are described herein. Suitable, non-immunogenic linker peptides are, for example, $(G_4S)_n$, $(SG_4)_n$ or $G_4(SG_4)_n$ peptide linkers, wherein “n” is generally a number between 1 and 10, typically between 1 and 4, in particular 2, i.e. the peptides selected from the group consisting of GGGGS (SEQ ID NO:109),

GGGSGGGGS (SEQ ID NO:110), SGGGSGGGG (SEQ ID NO:111), (G₄S)₃ or GGGSGGGSGGGGS (SEQ ID NO:112), GGGSGGGSGGGG or G₄(SG₄)₂ (SEQ ID NO:113), and (G₄S)₄ or GGGSGGGSGGGSGGGGS (SEQ ID NO:114), but also include the sequences GSPGSSSSGS (SEQ ID NO:115), GSGSGSGS (SEQ ID NO:116), GSGSGNGS (SEQ ID NO:117), GSGSGSG (SEQ ID NO:118), GSGSG (SEQ ID NO:119), GGSG (SEQ ID NO:120), GSGNGSG (SEQ ID NO:121), GGNGSGSG (SEQ ID NO:122) and GGNGSG (SEQ ID NO:123). Peptide linkers of particular interest are (G₄S)₁ or GGGGS (SEQ ID NO:109), (G₄S)₂ or GGGSGGGGS (SEQ ID NO:110), (G₄S)₃ or GGGSGGGSGGGGS (SEQ ID NO:112) and (G₄S)₄ or GGGSGGGSGGGSGGGGS (SEQ ID NO:114).

10 The term "**amino acid**" as used within this application denotes the group of naturally occurring carboxy α -amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S),
15 threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

A "**fusion polypeptide**" as used herein refers to a single chain polypeptide comprising a heavy chain variable domain (VH) and/or light chain variable domain (VL) of a moiety capable of specific binding to a costimulatory TNF receptor family member, fused to a trimerization domain. In some embodiments, the fusion polypeptide further comprises a VH and/or VL of a moiety capable of specific binding to a target cell antigen. The fusion may occur by direct linking of the N- or C-terminal amino acids of the respective domains via a peptide linker. By "fused" or "connected" it is meant that the components are linked by peptide bonds, either directly or via one or more peptide linkers.

In some embodiments, the fusion polypeptide comprises a scFv fragment capable of specific binding to a costimulatory TNF receptor family member (i.e. the VH and VL domains of a scFv fragment). In some embodiments, the fusion polypeptide comprises the VH and CH1 domains of a Fab fragment capable of binding to a costimulatory TNF receptor family member. In some embodiments, the fusion polypeptide comprises the VL and CL domains of a Fab fragment capable of binding to a costimulatory TNF receptor family member.

30 In some embodiments, the fusion polypeptide comprises a scFv fragment capable of specific binding to a target cell antigen (i.e. the VH and VL domains of a scFv fragment). In some embodiments, the fusion polypeptide comprises the VH and CH1 domains of a Fab fragment capable of binding to a target cell antigen. In some embodiments, the fusion polypeptide comprises the VL and CL domains of a Fab fragment capable of binding to a target
35 cell antigen.

The term “**trimerization domain**” refers to an amino acid sequence within a polypeptide that promotes self-assembly by associating with two other trimerization domains to form a trimer. The term is also used to refer to the polynucleotide encoding said amino acid sequence. Typically, the trimerization domain comprises an amino acid sequence able to form an alpha-helical coiled-coil domain or an isoleucine zipper domain. Suitable trimerization domains include TRAF2 (UniProt accession no. Q12933, SEQ ID NO:124), in particular amino acids 299 to 348 or amino acids 310 to 349), Thrombospondin 1 (UniProt accession no. P07996, SEQ ID NO:125), in particular amino acids 291 to 314), Matrilin-4 (UniProt accession no. O95460, SEQ ID NO:126), in particular amino acids 594 to 618; CMP (matrilin-1) (Uniprot accession No. P21941, SEQ ID NO:1), in particular amino acids 454 to 496, and Cubilin (UniProt accession no. O60494, SEQ ID NO: 127), in particular amino acids 104 to 138. An exemplary isoleucine zipper domain is the engineered yeast GCN4 isoleucine variant described by Harbury et al. (1993) Science 262, 1401- 1407 comprising the amino acid sequence of SEQ ID NO:128.

A particular trimerization domain is “**huCMP**” or “**CMP**” or “human cartilage matrix protein”, a protein that is also known as matrilin-1 or MATN1 or CRTM (UniProt accession no. P21941, SEQ ID NO:1). The term “huCMP trimerization domain” or “trimerization domain derived from human cartilage matrix protein (CMP)” refers to a polypeptide structure capable of associating with two similar or identical polypeptides to form a stable trimer. The trimerisation is mediated through ionic bonds and other non-covalent bonds formed between adjacent charged amino acids of the polypeptide chains. The huCMP trimerization domain has been described e.g. in Beck et al (1996), J. Mol. Biol. 256, 909–923. A huCMP trimerization domain of particular interest comprises a sequence having at least 95% identity and most preferably at least 98% identity to SEQ ID NO 2. In one embodiment said trimerization domain comprises the sequence of SEQ ID NO. 2.

“**Percent (%) amino acid sequence identity**” with respect to a reference polypeptide (protein) sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN. SAWI or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code

has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX
5 operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary. In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to,
10 with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid
15 sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

In certain embodiments, **amino acid sequence variants** of the trimeric antigen binding
20 molecules provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the trimeric antigen binding molecules. Amino acid sequence variants of the trimeric antigen binding molecules may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the molecules, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions
25 into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding. Sites of interest for substitutional mutagenesis include the HVRs and Framework (FRs). Conservative substitutions are provided in **Table B** under the heading "Preferred Substitutions" and further
30 described below in reference to amino acid side chain classes (1) to (6). Amino acid substitutions may be introduced into the molecule of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

Table B.

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

The term “**amino acid sequence variants**” includes substantial variants wherein there are amino acid substitutions in one or more hypervariable region residues of a parent antigen binding molecule (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antigen binding molecule and/or will have substantially retained certain biological properties of the parent antigen binding molecule. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antigen binding molecules displayed on phage and screened for a particular biological activity (e.g. binding affinity). In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antigen binding molecule to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antigen binding molecule complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include the fusion to the N- or C-terminus to a polypeptide which increases the serum half-life of the trimeric antigen binding molecules.

In certain aspects, the trimeric antigen binding molecules provided herein are altered to increase or decrease the extent to which the antibody is glycosylated. Glycosylation variants of the molecules may be conveniently obtained by altering the amino acid sequence such that one or more glycosylation sites is created or removed. In certain aspects, the trimeric antigen binding molecules provided herein may be further modified to contain additional non-proteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization

of the antigen binding molecule include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, propylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antigen binding molecule to be improved.

The term "**polynucleotide**" refers to an isolated nucleic acid molecule or construct, e.g. messenger RNA (mRNA), virally-derived RNA, or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (e.g. an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid molecule" refers to any one or more nucleic acid segments, e.g. DNA or RNA fragments, present in a polynucleotide.

By "**isolated**" nucleic acid molecule or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding a polypeptide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. An isolated polynucleotide includes a polynucleotide molecule contained in cells that ordinarily contain the polynucleotide molecule, but the polynucleotide molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the present invention, as well as positive and negative strand forms, and double-stranded forms. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

By a nucleic acid or polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the

polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of
5 nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence. As a practical matter, whether any
10 particular polynucleotide sequence is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs, such as the ones discussed above for polypeptides (e.g. ALIGN-2).

The term "**expression cassette**" refers to a polynucleotide generated recombinantly or
15 synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter. In certain
20 embodiments, the expression cassette of the invention comprises polynucleotide sequences that encode bispecific antigen binding molecules of the invention or fragments thereof.

The term "**vector**" or "expression vector" is synonymous with "expression construct" and refers to a DNA molecule that is used to introduce and direct the expression of a specific gene to which it is operably associated in a target cell. The term includes the vector as a self-replicating
25 nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. The expression vector of the present invention comprises an expression cassette. Expression vectors allow transcription of large amounts of stable mRNA. Once the expression vector is inside the target cell, the ribonucleic acid molecule or protein that is encoded by the gene is produced by the cellular transcription and/or translation machinery. In
30 one embodiment, the expression vector of the invention comprises an expression cassette that comprises polynucleotide sequences that encode bispecific antigen binding molecules of the invention or fragments thereof.

The terms "**host cell**", "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of
35 such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages.

Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein. A host cell is any type of cellular system that can be used to generate the bispecific antigen binding molecules of the present invention. Host cells include cultured cells, e.g. mammalian cultured cells, such as CHO cells, HEK cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue.

10 An "**effective amount**" of an agent refers to the amount that is necessary to result in a physiological change in the cell or tissue to which it is administered.

15 A "**therapeutically effective amount**" of an agent, e.g. a pharmaceutical composition, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. A therapeutically effective amount of an agent for example eliminates, decreases, delays, minimizes or prevents adverse effects of a disease.

 An "**individual**" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g. cows, sheep, cats, dogs, and horses), primates (e.g. humans and non-human primates such as monkeys), rabbits, and rodents (e.g. mice and rats). Particularly, the individual or subject is a human. In some embodiments, an individual or subject may be a patient.

20 The term "**pharmaceutical composition**" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

25 A "**pharmaceutically acceptable excipient**" refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable excipient includes, but is not limited to, a buffer, a stabilizer, or a preservative.

30 As used herein, "**treatment**" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

In some embodiments, the molecules of the invention are used to delay development of a disease or to slow the progression of a disease.

The term “**cancer**” as used herein refers to proliferative diseases, such as lymphomas, lymphocytic leukemias, lung cancer, non-small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwannomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma and Ewings sarcoma, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers.

Trimeric antigen binding molecules of the invention

The invention provides novel trimeric antigen binding molecules with particularly advantageous properties such as producibility, stability, binding affinity, biological activity, targeting efficiency and reduced toxicity.

The present invention provides a trimeric antigen binding molecule comprising three fusion polypeptides, each of the three fusion polypeptides comprising

- (a) a moiety capable of specific binding to a costimulatory TNF receptor family member,
- (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1, and
- (c) a moiety capable of specific binding to a target cell antigen.

Thus, disclosed herein is a trimeric antigen binding molecule comprising three fusion polypeptides, each of the three fusion polypeptides comprising

- (a) a moiety capable of specific binding to a costimulatory TNF receptor family member,
- (b) a trimerization domain, and
- (c) a moiety capable of specific binding to a target cell antigen.

A trimerization domain can be derived from a polypeptide derived from a protein selected from the group consisting of human TRAF2, human thrombospondin 1, human matrilin-4,

human cartilage matrix protein (huCMP) and human cubilin. It can also be an isoleucine zipper domain, for example the peptide with the amino acid sequence of SEQ ID NO:128. A trimeric antigen binding molecule of the invention comprises a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1.

5 The trimerization domain derived from human cartilage matrix protein (huCMP) comprises at least a part of SEQ ID NO.: 1. In one aspect, the trimerization domain comprises an amino acid sequence having at least 95% identity and most preferably at least 98% identity to SEQ ID NO:2. More particularly, the trimerization domain comprises the amino acid sequence of SEQ ID NO:2. The trimerization domain derived from human cartilage matrix protein (CMP) is
10 herein further referred to as “huCMP trimerization domain”.

The trimeric antigen binding molecule according to the present invention may associate to form trimers through disulphide bonds formed between the trimerisation domains of the fusion polypeptides.

15 In a particular aspect, the trimeric antigen binding molecule comprises three fusion polypeptides, each of the three fusion polypeptides comprising a trimerization domain comprising an amino acid sequence having at least 95% identity and in particular at least 98% identity to SEQ ID NO:2. More particularly, each of the three fusion polypeptides comprises one trimerization domain comprising the amino acid sequence of SEQ ID NO:2.

20 In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member is selected from the group consisting of is selected from the group consisting of an antibody, an antibody fragment and a scaffold antigen binding protein. In one aspect, the moiety capable of specific binding to a costimulatory TNF receptor family member is selected from the group consisting of an antibody fragment, a Fab molecule, a crossover Fab molecule, a single chain Fab molecule, a Fv molecule, a scFv molecule, a single domain
25 antibody, an aVH and a scaffold antigen binding protein.

30 In some embodiments, the invention provides a trimeric antigen binding molecule as defined herein before, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member is an antibody fragment. In particular, the antibody fragment is selected from the group consisting of a Fab molecule, a crossover Fab molecule, a single chain Fab molecule, a Fv molecule, a scFv molecule, a single domain antibody, and aVH. In one aspect, the moiety capable of specific binding to a target cell antigen is a single chain Fab molecule. In one aspect, the moiety capable of specific binding to a target cell antigen is a single domain antibody or an aVH.

In a further aspect, the invention provides a trimeric antigen binding molecule as defined herein before, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member is a scaffold antigen binding protein. In particular, the moiety capable of specific binding to a costimulatory TNF receptor family member can be a specifically designed ankyrin repeat protein.

In a particular aspect, the invention is concerned with a trimeric antigen binding molecule as defined herein before, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member is a Fab molecule capable of specific binding to a costimulatory TNF receptor family member. Thus, the invention provides an antigen binding molecule comprising

(a) a Fab molecule capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1, and (c) a moiety capable of specific binding to a target cell antigen.

In a further aspect, said Fab molecule is fused at the C-terminal amino acid of the CH1 domain to the N-terminal amino acid of the trimerization domain, optionally through a peptide linker as defined herein.

In some embodiments, the costimulatory TNF receptor family member is selected from the group consisting of 4-1BB, OX40 and GITR. More particularly the costimulatory TNF receptor family member is selected from OX40 and 4-1BB.

In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member binds to OX40. In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:3.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety comprises a VH comprising

(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5,

(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14,

and a VL comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17,

- (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, and
- (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25 and SEQ ID NO:26.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO: 29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37 and SEQ ID NO:39 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO: 30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 and SEQ ID NO:40.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises

(a) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:4, CDR-H2 comprising the amino acid sequence of SEQ ID NO:6, CDR-H3 comprising the amino acid sequence of SEQ ID NO:8 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:15, CDR-L2 comprising the amino acid sequence of SEQ ID NO:18 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:21,

(b) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:4, CDR-H2 comprising the amino acid sequence of SEQ ID NO:6, CDR-H3 comprising the amino acid sequence of SEQ ID NO:9 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:15, CDR-L2 comprising the amino acid sequence of SEQ ID NO:18 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:22,

(c) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:4, CDR-H2 comprising the amino acid sequence of SEQ ID NO:6, CDR-H3 comprising the amino acid sequence of SEQ ID NO:10 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:15, CDR-L2 comprising the amino acid sequence of SEQ ID NO:18 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:23,

(d) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:4, CDR-H2 comprising the amino acid sequence of SEQ ID NO:6, CDR-H3 comprising the amino acid sequence of SEQ ID NO:11 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:18, CDR-L2 comprising the amino acid sequence of SEQ ID NO:16 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:24,

(e) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:5, CDR-H2 comprising the amino acid sequence of SEQ ID NO:7, CDR-H3 comprising the amino acid sequence of SEQ ID NO:12 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:16, CDR-L2 comprising the amino acid sequence of SEQ ID NO:17 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:25,

(f) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:5, CDR-H2 comprising the amino acid sequence of SEQ ID NO:7, CDR-H3 comprising the amino acid sequence of SEQ ID NO:13 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:16, CDR-L2 comprising the amino acid sequence of SEQ ID NO:17 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:25, or

(g) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:5, CDR-H2 comprising the amino acid sequence of SEQ ID NO:7, CDR-H3 comprising the amino acid sequence of SEQ ID NO:14 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:17, CDR-L2 comprising the amino acid sequence of SEQ ID NO:18 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:26.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:27 and a VL comprising the amino acid sequence of SEQ ID NO:28,

(ii) a VH comprising the amino acid sequence of SEQ ID NO:29 and a VL comprising the amino acid sequence of SEQ ID NO:30,

(iii) a VH comprising the amino acid sequence of SEQ ID NO:31 and a VL comprising the amino acid sequence of SEQ ID NO:32,

(iv) a VH comprising the amino acid sequence of SEQ ID NO:33 and a VL comprising the amino acid sequence of SEQ ID NO:34,

(v) a VH comprising the amino acid sequence of SEQ ID NO:35 and a VL comprising the amino acid sequence of SEQ ID NO:36,

(vi) a VH comprising the amino acid sequence of SEQ ID NO:37 and a VL comprising the amino acid sequence of SEQ ID NO:38, or

(vii) a VH comprising the amino acid sequence of SEQ ID NO:39 and a VL comprising the amino acid sequence of SEQ ID NO:40.

In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member binds to 4-1BB. In some embodiments, the moiety capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:41.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety comprises a VH comprising

(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:42 and SEQ ID NO:43,

5 (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50,

and a VL comprising

10 (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:51 and SEQ ID NO:52,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:53 and SEQ ID NO:54, and

15 (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58 and SEQ ID NO:59.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66 and SEQ ID NO:68 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67 and SEQ ID NO:69.

25 In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises

(a) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:42, CDR-H2 comprising the amino acid sequence of SEQ ID NO:44, CDR-H3 comprising the amino acid sequence of SEQ ID NO:46 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:51, CDR-L2 comprising the amino acid sequence of SEQ ID NO:53 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:55,

30 (b) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:43, CDR-H2 comprising the amino acid sequence of SEQ ID NO:45, CDR-H3 comprising the amino acid sequence of SEQ ID NO:47 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:52, CDR-L2 comprising the amino acid sequence of SEQ ID NO:54 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:56,

(c) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:42, CDR-H2 comprising the amino acid sequence of SEQ ID NO:44, CDR-H3 comprising the amino acid sequence of SEQ ID NO:48 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:51, CDR-L2 comprising the amino acid sequence of SEQ ID NO:53 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:57,

(d) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:42, CDR-H2 comprising the amino acid sequence of SEQ ID NO:44, CDR-H3 comprising the amino acid sequence of SEQ ID NO:49 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:51, CDR-L2 comprising the amino acid sequence of SEQ ID NO:53 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:58, or

(e) a VH comprising CDR-H1 comprising the amino acid sequence of SEQ ID NO:42, CDR-H2 comprising the amino acid sequence of SEQ ID NO:44, CDR-H3 comprising the amino acid sequence of SEQ ID NO:50 and a VL comprising CDR-L1 comprising the amino acid sequence of SEQ ID NO:51, CDR-L2 comprising the amino acid sequence of SEQ ID NO:53 and CDR-L3 comprising the amino acid sequence of SEQ ID NO:59.

In some embodiments, the trimeric antigen binding molecule comprises a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:60 and a VL comprising the amino acid sequence of SEQ ID NO:61,

(ii) a VH comprising the amino acid sequence of SEQ ID NO:62 and a VL comprising the amino acid sequence of SEQ ID NO:63,

(iii) a VH comprising the amino acid sequence of SEQ ID NO:64 and a VL comprising the amino acid sequence of SEQ ID NO:65,

(iv) a VH comprising the amino acid sequence of SEQ ID NO:66 and a VL comprising the amino acid sequence of SEQ ID NO:67, or

(v) a VH comprising the amino acid sequence of SEQ ID NO:68 and a VL comprising the amino acid sequence of SEQ ID NO:69.

In another aspect, provided is a trimeric antigen binding molecule comprising three fusion polypeptides as described before, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member is fused at the C-terminal amino acid to the N-terminal amino acid of the trimerization domain, optionally through a peptide linker. The fusion can be a direct bond between the moiety capable of specific binding to a costimulatory TNF receptor family member and the trimerization domain, or the moiety capable of specific binding to a costimulatory TNF receptor family member and the trimerization domain may be connected through a peptide linker. In a particular aspect, the moiety capable of specific binding to a

costimulatory TNF receptor family member and the trimerization domain are connected through a peptide linker.

Typically, the peptide linker is a peptide comprising 2 to 20 amino acids. In particular, the peptide linker is a peptide selected from the group consisting of GGGGS (SEQ ID NO:109),
 5 GGGGSGGGGS (SEQ ID NO:110), SGGGSGGGG (SEQ ID NO:111), $(G_4S)_3$ or
 GGGGSGGGGSGGGGS (SEQ ID NO:112), GGGGSGGGGSGGGG or $G_4(SG_4)_2$ (SEQ ID
 NO:113), and $(G_4S)_4$ or GGGGSGGGGSGGGGSGGGGS (SEQ ID NO:114), but also include
 the sequences GSPGSSSSGS (SEQ ID NO:115), GSGSGSGS (SEQ ID NO:116), GSGSGNGS
 (SEQ ID NO:117), GSGSGSG (SEQ ID NO:118), GSGSG (SEQ ID NO:119), GGSG (SEQ
 10 ID NO:120), GSGNGSG (SEQ ID NO:121), GGNGSGSG (SEQ ID NO:122) and GGNGSG
 (SEQ ID NO:123). More particularly, the peptide linker is selected from $(G_4S)_1$ or GGGGS
 (SEQ ID NO:109), $(G_4S)_2$ or GGGGSGGGGS (SEQ ID NO:110), $(G_4S)_3$ or
 GGGGSGGGGSGGGGS (SEQ ID NO:112) and $(G_4S)_4$ or GGGGSGGGGSGGGGSGGGGS
 (SEQ ID NO:114). Most particularly, the moiety capable of specific binding to a costimulatory
 15 TNF receptor family member and the huCMP trimerization domain are connected by a peptide
 linker of SEQ ID NO:114.

In a further aspect, provided is a trimeric antigen binding molecule comprising three fusion
 polypeptides as described before, wherein the moiety capable of specific binding to a target cell
 antigen is fused at the N-terminal amino acid to the C-terminal amino acid of the trimerization
 20 domain, optionally through a peptide linker. In a particular aspect, the trimerization domain and
 the moiety capable of specific binding to a target cell antigen are connected through a peptide
 linker.

In particular, the peptide linker is a peptide selected from the group consisting of GGGGS
 (SEQ ID NO:109), GGGGSGGGGS (SEQ ID NO:110), SGGGSGGGG (SEQ ID NO:111),
 25 $(G_4S)_3$ or GGGGSGGGGSGGGGS (SEQ ID NO:112), GGGGSGGGGSGGGG or $G_4(SG_4)_2$
 (SEQ ID NO:113), and $(G_4S)_4$ or GGGGSGGGGSGGGGSGGGGS (SEQ ID NO:114), but also
 include the sequences GSPGSSSSGS (SEQ ID NO:115), GSGSGSGS (SEQ ID NO:116),
 GSGSGNGS (SEQ ID NO:117), GSGSGSG (SEQ ID NO:118), GSGSG (SEQ ID NO:119),
 GGSG (SEQ ID NO:120), GSGNGSG (SEQ ID NO:121), GGNGSGSG (SEQ ID NO:122)
 30 and GGNGSG (SEQ ID NO:123). More particularly, the peptide linker is selected from $(G_4S)_1$
 or GGGGS (SEQ ID NO:109), $(G_4S)_2$ or GGGGSGGGGS (SEQ ID NO:110), $(G_4S)_3$ or
 GGGGSGGGGSGGGGS (SEQ ID NO:112) and $(G_4S)_4$ or GGGGSGGGGSGGGGSGGGGS
 (SEQ ID NO:114). Most particularly, the huCMP trimerization domain and the moiety capable
 of specific binding to a target cell antigen are connected by a peptide linker of SEQ ID NO:110.

In another aspect, the invention provides a trimeric antigen binding molecule comprising three fusion polypeptides as described before, wherein the moiety capable of specific binding to a target cell antigen is selected from the group consisting of an antibody, an antibody fragment and a scaffold antigen binding protein. In one aspect, the moiety capable of specific binding to a target cell antigen is selected from the group consisting of an antibody fragment, a Fab molecule, a crossover Fab molecule, a single chain Fab molecule, a Fv molecule, a scFv molecule, a single domain antibody, an aVH and a scaffold antigen binding protein.

In one aspect, the invention provides a trimeric antigen binding molecule as defined herein before, wherein the moiety capable of specific binding to a target cell antigen is an antibody fragment. In particular, the antibody fragment is selected from the group consisting of a Fab molecule, a crossover Fab molecule, a single chain Fab molecule, a Fv molecule, a scFv molecule, a single domain antibody, and aVH. In one aspect, the moiety capable of specific binding to a target cell antigen is a single chain Fab molecule. In one aspect, the moiety capable of specific binding to a target cell antigen is a single domain antibody or an aVH.

In a further aspect, the invention provides a trimeric antigen binding molecule as defined herein before, wherein the moiety capable of specific binding to a target cell antigen is a scaffold antigen binding protein. In particular, the moiety capable of specific binding to a target cell antigen can be a specifically designed ankyrin repeat protein.

In a particular aspect, the invention is concerned with a trimeric antigen binding molecule as defined herein before, wherein the moiety capable of specific binding to a target cell antigen is a Fab molecule capable of specific binding to a target cell antigen. Thus, the invention provides an antigen binding molecule comprising (a) a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1, and (c) a Fab molecule capable of specific binding to a target cell antigen.

In a further aspect, said Fab molecule is fused at the C-terminal amino acid of the CH1 domain to the N-terminal amino acid of the trimerization domain, optionally through a peptide linker as defined above.

In a further aspect, provided is a trimeric antigen binding molecule comprising three fusion polypeptides as described before, wherein the target cell antigen is selected from the group consisting of Fibroblast Activation Protein (FAP), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), Carcinoembryonic Antigen (CEA), CD19, CD20 and CD33. In a particular aspect, the target cell antigen is Fibroblast

Activation Protein (FAP). In some embodiments, the moiety capable of specific binding to a target cell antigen binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:86.

In a further aspect, provided is a trimeric antigen binding molecule comprising three fusion polypeptides as described before, wherein the moiety capable of specific binding to FAP

5 comprises

(a) a VH comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:70, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:71 and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:72, and a VL comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:73, (v) CDR-L2 comprising the amino acid sequence of

10 SEQ ID NO:74 and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:75 or

(b) a VH comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:76, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:77 and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:78, and a VL comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:79, (v) CDR-L2 comprising the amino acid sequence of

15 SEQ ID NO:80 and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:81.

In a further aspect, the moiety capable of specific binding to FAP comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:82 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid

20 sequence of SEQ ID NO:83.

In another aspect, the moiety capable of specific binding to FAP comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:84 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid

25 sequence of SEQ ID NO:85.

In a further aspect, the moiety capable of specific binding to FAP comprises

(a) a VH comprising the amino acid sequence of SEQ ID NO:82 and a VL comprising the amino acid sequence of SEQ ID NO:83, or (b) a VH comprising the amino acid sequence of SEQ ID NO:84 and a VL comprising the amino acid sequence of SEQ ID NO:85.

30 In a further aspect, provided is a trimeric antigen binding molecule comprising three fusion polypeptides as described before, wherein said three fusion polypeptides are identical.

In some embodiments, the trimeric antigen binding molecules of the present invention are characterized by agonistic binding to a costimulatory TNF receptor family member. Particularly,

the costimulatory TNF receptor family member is selected from the group consisting of OX40 and 4-1BB.

In another aspect, the invention provides a fusion polypeptide comprising (a) a VH and/or VL of a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) comprising the amino acid sequence of SEQ ID NO:2, wherein the trimerization domain is capable of mediating stable association of the fusion polypeptide with two further such fusion polypeptides and (c) a VH and/or VL of a moiety capable of specific binding to a target cell antigen.

In some embodiments the fusion polypeptide comprises a VH of a moiety capable of specific binding to FAP, wherein the VH comprises

- (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:70 and SEQ ID NO:76,
- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:71 and SEQ ID NO:77, and
- (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:72 and SEQ ID NO:78.

In some embodiments the fusion polypeptide comprises a VH of a moiety capable of specific binding to FAP, wherein the VH comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:82 and SEQ ID NO:84.

In some embodiments the fusion polypeptide comprises a VL of a moiety capable of specific binding to FAP, wherein the VL comprises

- (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:73 and SEQ ID NO:79,
- (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:74 and SEQ ID NO:80, and
- (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75 and SEQ ID NO:81.

In some embodiments, the fusion polypeptide comprises a VL of a moiety capable of specific binding to FAP, wherein the VL comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:83 and SEQ ID NO:85.

In some embodiments, the fusion polypeptide comprises a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises

- (i) a VH comprising the amino acid sequence of SEQ ID NO:82 and a VL comprising the amino acid sequence of SEQ ID NO:83, or
- (ii) a VH comprising the amino acid sequence of SEQ ID NO:84 and a VL comprising the amino acid sequence of SEQ ID NO:85.

5 In some embodiments the fusion polypeptide comprises a VH of a moiety capable of specific binding to OX40, wherein the moiety comprises a VH comprising

- (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5,
- 10 (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and
- (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14.

15 In some embodiments the fusion polypeptide comprises a VH of a moiety capable of specific binding to OX40, wherein the VH comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO: 29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37 and SEQ ID NO:39.

20 In some embodiments the fusion polypeptide comprises a VL of a moiety capable of specific binding to OX40, wherein the moiety comprises a VL comprising

- (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17,
- (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, and
- 25 (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25 and SEQ ID NO:26.

30 In some embodiments, the fusion polypeptide comprises a VL of a moiety capable of specific binding to OX40, wherein the VL comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO: 30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 and SEQ ID NO:40.

In some embodiments, the fusion polypeptide comprises comprises a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises

- (i) a VH comprising the amino acid sequence of SEQ ID NO:27 and a VL comprising the amino acid sequence of SEQ ID NO:28,
- (ii) a VH comprising the amino acid sequence of SEQ ID NO:29 and a VL comprising the amino acid sequence of SEQ ID NO:30,
- 5 (iii) a VH comprising the amino acid sequence of SEQ ID NO:31 and a VL comprising the amino acid sequence of SEQ ID NO:32,
- (iv) a VH comprising the amino acid sequence of SEQ ID NO:33 and a VL comprising the amino acid sequence of SEQ ID NO:34,
- 10 (v) a VH comprising the amino acid sequence of SEQ ID NO:35 and a VL comprising the amino acid sequence of SEQ ID NO:36,
- (vi) a VH comprising the amino acid sequence of SEQ ID NO:37 and a VL comprising the amino acid sequence of SEQ ID NO:38, or
- (vii) a VH comprising the amino acid sequence of SEQ ID NO:39 and a VL comprising the amino acid sequence of SEQ ID NO:40.

15 In particular embodiments, the present invention provides a fusion polypeptide comprising, or consisting of, the amino acid selected from the group consisting of SEQ ID NO:227, 229, 231, 233, 235 and 237.

In some embodiments the fusion polypeptide comprises a VH of a moiety capable of specific binding to 4-1BB, wherein the moiety comprises a VH comprising

- 20 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:42 and SEQ ID NO:43,
- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and
- 25 (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50.

In some embodiments the fusion polypeptide comprises a VH of a moiety capable of specific binding to 4-1BB, wherein the VH comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66 and SEQ
30 ID NO:68.

In some embodiments the fusion polypeptide comprises a VL of a moiety capable of specific binding to 4-1BB, wherein the moiety comprises a VL comprising

- (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:51 and SEQ ID NO:52,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:53 and SEQ ID NO:54, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58 and SEQ ID NO:59.

5 In some embodiments, the fusion polypeptide comprises a VL of a moiety capable of specific binding to 4-1BB, wherein the VL comprises an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67 and SEQ ID NO:69.

10 In some embodiments, the fusion polypeptide comprises comprises a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:60 and a VL comprising the amino acid sequence of SEQ ID NO:61,

(ii) a VH comprising the amino acid sequence of SEQ ID NO:62 and a VL comprising the amino acid sequence of SEQ ID NO:63,

(iii) a VH comprising the amino acid sequence of SEQ ID NO:64 and a VL comprising the amino acid sequence of SEQ ID NO:65,

(iv) a VH comprising the amino acid sequence of SEQ ID NO:66 and a VL comprising the amino acid sequence of SEQ ID NO:67, or

(v) a VH comprising the amino acid sequence of SEQ ID NO:68 and a VL comprising the amino acid sequence of SEQ ID NO:69.

In particular embodiments, the present invention provides a fusion polypeptide comprising, or consisting of, the amino acid selected from the group consisting of SEQ ID NO:246, 248, 250 and 252.

25 Polynucleotides

The invention further provides isolated polynucleotides encoding a trimeric antigen binding molecule of the invention as described herein, or a fragment thereof.

The isolated polynucleotides encoding trimeric antigen binding molecules of the invention may be expressed as a single polynucleotide that encodes the entire antigen binding molecule or as multiple (e.g., two or more) polynucleotides that are co-expressed. Polypeptides encoded by polynucleotides that are co-expressed may associate through, e.g., disulfide bonds or other means to form a functional trimeric antigen binding molecule. For example, the light chain portion of a moiety capable of specific binding to a target cell antigen may be encoded by a separate polynucleotide from the heavy chain portion of the capable of specific binding to a

target cell antigen. When co-expressed, the heavy chain polypeptides will associate with the light chain polypeptides to form the moiety capable of specific binding to a target cell antigen.

Similarly, the light chain portion of a moiety capable of specific binding to a costimulatory TNF receptor family member may be encoded by a separate polynucleotide from the heavy chain

5 portion of the capable of specific binding to a costimulatory TNF receptor family member. When co-expressed, the heavy chain polypeptides will associate with the light chain polypeptides to form the moiety capable of specific binding to a costimulatory TNF receptor family member.

In a particular aspect, the invention relates to an isolated polynucleotide encoding a fusion polypeptide of the present invention as described herein.

10 In particular embodiments, the present invention provides a polynucleotide encoding a fusion polypeptide comprising, or consisting of, the amino acid selected from the group consisting of SEQ ID NO:227, 229, 231, 233, 235 and 237. More particularly, provided is a polynucleotide comprising, or consisting of, the sequence selected from the group consisting of SEQ ID NO:215, 217, 219, 221, 223 and 225.

15 In particular embodiments, the present invention provides a polynucleotide encoding a fusion polypeptide comprising, or consisting of, the amino acid selected from the group consisting of SEQ ID NO: 246, 248, 250 and 252. More particularly, provided is a polynucleotide comprising, or consisting of, the sequence selected from the group consisting of SEQ ID NO:238, 240, 242 and 244.

20 In certain embodiments the polynucleotide or nucleic acid is DNA. In other embodiments, a polynucleotide of the present invention is RNA, for example, in the form of messenger RNA (mRNA). RNA of the present invention may be single stranded or double stranded.

25 According to another aspect of the invention, there is provided an isolated polynucleotide encoding a trimeric antigen binding molecule as defined herein before or a fusion polypeptide as described herein before. The invention further provides a vector, particularly an expression vector, comprising the isolated polynucleotide of the invention and a host cell comprising the isolated polynucleotide or the vector of the invention. In some embodiments the host cell is a eukaryotic cell, particularly a mammalian cell.

30 In another aspect, provided is a method for producing the trimeric antigen binding molecule of the invention, comprising the steps of (i) culturing the host cell of the invention under conditions suitable for expression of said antigen binding molecule, and (ii) isolating said trimeric antigen binding molecule. The invention also encompasses a trimeric antigen binding molecule produced by the method of the invention.

Recombinant Methods

Trimeric antigen binding molecules of the invention may be obtained, for example, by solid-state peptide synthesis (e.g. Merrifield solid phase synthesis) or recombinant production. For recombinant production one or more polynucleotide encoding the antigen binding molecule or polypeptide fragments thereof, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such polynucleotide may be readily isolated and sequenced using conventional procedures. In one aspect of the invention, a vector, preferably an expression vector, comprising one or more of the polynucleotides of the invention is provided. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of the trimeric antigen binding molecule (fragment) along with appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, Cold Spring Harbor Laboratory, N.Y. (1989); and Ausubel et al., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, Greene Publishing Associates and Wiley Interscience, N.Y. (1989). The expression vector can be part of a plasmid, virus, or may be a nucleic acid fragment. The expression vector includes an expression cassette into which the polynucleotide encoding the trimeric antigen binding molecule or polypeptide fragments thereof (i.e. the coding region) is cloned in operable association with a promoter and/or other transcription or translation control elements. As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, if present, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, 5' and 3' untranslated regions, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, e.g. on a single vector, or in separate polynucleotide constructs, e.g. on separate (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, e.g. a vector of the present invention may encode one or more polypeptides, which are post- or co-translationally separated into the final proteins via proteolytic cleavage. In addition, a vector, polynucleotide, or nucleic acid of the invention may encode heterologous coding regions, either fused or unfused to a polynucleotide encoding the trimeric antigen binding molecule of the invention or polypeptide fragments thereof, or variants or derivatives thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain. An operable association is when a coding region for a gene product, e.g. a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory

sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory
5 sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for
10 example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription.

Suitable promoters and other transcription control regions are disclosed herein. A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions, which function in vertebrate cells, such as, but not
15 limited to, promoter and enhancer segments from cytomegaloviruses (e.g. the immediate early promoter, in conjunction with intron-A), simian virus 40 (e.g. the early promoter), and retroviruses (such as, e.g. Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit α -globin, as well as other sequences capable of controlling gene expression in eukaryotic
20 cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as inducible promoters (e.g. promoters inducible tetracyclins). Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from viral systems (particularly an internal ribosome entry site, or
25 IRES, also referred to as a CITE sequence). The expression cassette may also include other features such as an origin of replication, and/or chromosome integration elements such as retroviral long terminal repeats (LTRs), or adeno-associated viral (AAV) inverted terminal repeats (ITRs).

Polynucleotide and nucleic acid coding regions of the present invention may be associated
30 with additional coding regions which encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide of the present invention. For example, if secretion of the trimeric antigen binding molecule or polypeptide fragments thereof is desired, DNA encoding a signal sequence may be placed upstream of the nucleic acid encoding a trimeric antigen binding molecule of the invention or polypeptide fragments thereof. According to the
35 signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are

aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the translated polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, e.g. an immunoglobulin heavy chain or light chain signal peptide is used, or a functional
5 derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse β -glucuronidase.

10 DNA encoding a short protein sequence that could be used to facilitate later purification (e.g. a histidine tag) or assist in labeling the fusion protein may be included within or at the ends of the polynucleotide encoding a trimeric antigen binding molecule of the invention or polypeptide fragments thereof.

In a further aspect of the invention, a host cell comprising one or more polynucleotides of
15 the invention is provided. In certain embodiments a host cell comprising one or more vectors of the invention is provided. The polynucleotides and vectors may incorporate any of the features, singly or in combination, described herein in relation to polynucleotides and vectors, respectively. In one aspect, a host cell comprises (e.g. has been transformed or transfected with) a vector comprising a polynucleotide that encodes (part of) a trimeric antigen binding molecule
20 of the invention. As used herein, the term "host cell" refers to any kind of cellular system which can be engineered to generate the fusion proteins of the invention or fragments thereof. Host cells suitable for replicating and for supporting expression of antigen binding molecules are well known in the art. Such cells may be transfected or transduced as appropriate with the particular expression vector and large quantities of vector containing cells can be grown for seeding large
25 scale fermenters to obtain sufficient quantities of the antigen binding molecule for clinical applications. Suitable host cells include prokaryotic microorganisms, such as *E. coli*, or various eukaryotic cells, such as Chinese hamster ovary cells (CHO), human embryonic kidney (HEK) cells, insect cells, or the like. For example, polypeptides may be produced in bacteria in particular when glycosylation is not needed. After expression, the polypeptide may be isolated
30 from the bacterial cell paste in a soluble fraction and can be further purified. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized", resulting in the production of a polypeptide with a partially or fully human glycosylation pattern. See Gerngross, *Nat Biotech* 22, 1409-1414
35 (2004), and Li et al., *Nat Biotech* 24, 210-215 (2006).

Suitable host cells for the expression of (glycosylated) polypeptides are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures can also be utilized as hosts. See e.g. US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIESTM technology for producing antibodies in transgenic plants). Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293T cells as described, e.g., in Graham et al., J Gen Virol 36, 59 (1977)), baby hamster kidney cells (BHK), mouse sertoli cells (TM4 cells as described, e.g., in Mather, Biol Reprod 23, 243-251 (1980)), monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical carcinoma cells (HELA), canine kidney cells (MDCK), buffalo rat liver cells (BRL 3A), human lung cells (W138), human liver cells (Hep G2), mouse mammary tumor cells (MMT 060562), TRI cells (as described, e.g., in Mather et al., Annals N.Y. Acad Sci 383, 44-68 (1982)), MRC 5 cells, and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including dhfr- CHO cells (Urlaub et al., Proc Natl Acad Sci USA 77, 4216 (1980)); and myeloma cell lines such as YO, NS0, P3X63 and Sp2/0. For a review of certain mammalian host cell lines suitable for protein production, see, e.g., Yazaki and Wu, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003). Host cells include cultured cells, e.g., mammalian cultured cells, yeast cells, insect cells, bacterial cells and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. In one embodiment, the host cell is a eukaryotic cell, preferably a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell, a human embryonic kidney (HEK) cell or a lymphoid cell (e.g., YO, NS0, Sp20 cell). Standard technologies are known in the art to express foreign genes in these systems. Cells expressing a polypeptide comprising either the heavy or the light chain of an antigen binding domain, may be engineered so as to also express the other of the immunoglobulin chains such that the expressed product is an antigen binding domain that has both a heavy and a light chain.

In another aspect, provided is a method for producing the trimeric antigen binding molecule of the invention, comprising the steps of (i) culturing the host cell of the invention under conditions suitable for expression of said antigen binding molecule, and (ii) isolating said trimeric antigen binding molecule from the host cell or host cell culture medium.

The components of the trimeric antigen binding molecule are genetically fused to each other. Trimeric antigen binding molecules can be designed such that its components are fused directly to each other or indirectly through a linker sequence. The composition and length of the

linker may be determined in accordance with methods well known in the art and may be tested for efficacy. Examples of linker sequences between different components of trimeric antigen binding molecules are found in the sequences provided herein. Additional sequences may also be included to incorporate a cleavage site to separate the individual components of the fusion if
5 desired, for example an endopeptidase recognition sequence.

In certain embodiments the moieties capable of specific binding to a target cell antigen (e.g. Fab fragments or scFv) forming part of the antigen binding molecule comprise at least an immunoglobulin variable region capable of binding to a target cell antigen. Similarly, in certain
10 embodiments, the moieties capable of specific binding to a costimulatory TNF receptor family member (e.g. Fab fragments or scFv) forming part of the antigen binding molecule comprise at least an immunoglobulin variable region capable of binding to a costimulatory TNF receptor family member. Variable regions can form part of and be derived from naturally or non-naturally occurring antibodies and fragments thereof. Methods to produce polyclonal antibodies and
monoclonal antibodies are well known in the art (see e.g. Harlow and Lane, "Antibodies, a
15 laboratory manual", Cold Spring Harbor Laboratory, 1988). Non-naturally occurring antibodies can be constructed using solid phase-peptide synthesis, can be produced recombinantly (e.g. as described in U.S. patent No. 4,186,567) or can be obtained, for example, by screening combinatorial libraries comprising variable heavy chains and variable light chains (see e.g. U.S. Patent. No. 5,969,108 to McCafferty).

Any animal species of immunoglobulin can be used in the invention. Non-limiting immunoglobulins useful in the present invention can be of murine, primate, or human origin. If the fusion protein is intended for human use, a chimeric form of immunoglobulin may be used wherein the constant regions of the immunoglobulin are from a human. A humanized or fully human form of the immunoglobulin can also be prepared in accordance with methods well
20 known in the art (see e. g. U.S. Patent No. 5,565,332 to Winter). Humanization may be achieved by various methods including, but not limited to (a) grafting the non-human (e.g., donor antibody) CDRs onto human (e.g. recipient antibody) framework and constant regions with or without retention of critical framework residues (e.g. those that are important for retaining good antigen binding affinity or antibody functions), (b) grafting only the non-human specificity-determining
25 regions (CDRs or a-CDRs; the residues critical for the antibody-antigen interaction) onto human framework and constant regions, or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, Front Biosci 13, 1619-1633 (2008), and are further described, e.g., in Riechmann et al., Nature 332,
30 323-329 (1988); Queen et al., Proc Natl Acad Sci USA 86, 10029-10033 (1989); US Patent Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Jones et al., Nature 321, 522-525 (1986); Morrison et al., Proc Natl Acad Sci 81, 6851-6855 (1984); Morrison and Oi, Adv Immunol 44,

65-92 (1988); Verhoeyen et al., *Science* 239, 1534-1536 (1988); Padlan, *Molec Immun* 31(3), 169-217 (1994); Kashmiri et al., *Methods* 36, 25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol Immunol* 28, 489-498 (1991) (describing “resurfacing”); Dall’Acqua et al., *Methods* 36, 43-60 (2005) (describing “FR shuffling”); and Osbourn et al., *Methods* 36, 61-68 (2005) and
5 Klimka et al., *Br J Cancer* 83, 252-260 (2000) (describing the “guided selection” approach to FR shuffling). Particular immunoglobulins according to the invention are human immunoglobulins. Human antibodies and human variable regions can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr Opin Pharmacol* 5, 368-74 (2001) and Lonberg, *Curr Opin Immunol* 20, 450-459 (2008). Human
10 variable regions can form part of and be derived from human monoclonal antibodies made by the hybridoma method (see e.g. *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Human antibodies and human variable regions may also be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in
15 response to antigenic challenge (see e.g. Lonberg, *Nat Biotech* 23, 1117-1125 (2005)). Human antibodies and human variable regions may also be generated by isolating Fv clone variable region sequences selected from human-derived phage display libraries (see e.g., Hoogenboom et al. in *Methods in Molecular Biology* 178, 1-37 (O’Brien et al., ed., Humana Press, Totowa, NJ, 2001); and McCafferty et al., *Nature* 348, 552-554; Clackson et al., *Nature* 352, 624-628 (1991)).
20 Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments.

In certain aspects, the moieties capable of specific binding to the relevant target (e.g. Fab fragments or scFv) comprised in the antigen binding molecules of the present invention are engineered to have enhanced binding affinity according to, for example, the methods disclosed in
25 PCT publication WO 2012/020006 (see Examples relating to affinity maturation) or U.S. Pat. Appl. Publ. No. 2004/0132066. The ability of the antigen binding molecules of the invention to bind to a specific antigenic determinant can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. surface plasmon resonance technique (Liljeblad, et al., *Glyco J* 17, 323-329 (2000)), and traditional
30 binding assays (Heeley, *Endocr Res* 28, 217-229 (2002)). Competition assays may be used to identify an antigen binding molecule that competes with a reference antibody for binding to a particular antigen. In certain embodiments, such a competing antigen binding molecule binds to the same epitope (e.g. a linear or a conformational epitope) that is bound by the reference antigen binding molecule. Detailed exemplary methods for mapping an epitope to which an antigen
35 binding molecule binds are provided in Morris (1996) “Epitope Mapping Protocols”, in *Methods in Molecular Biology* vol. 66 (Humana Press, Totowa, NJ). In an exemplary competition assay, immobilized antigen is incubated in a solution comprising a first labeled antigen binding

molecule that binds to the antigen and a second unlabeled antigen binding molecule that is being tested for its ability to compete with the first antigen binding molecule for binding to the antigen. The second antigen binding molecule may be present in a hybridoma supernatant. As a control, immobilized antigen is incubated in a solution comprising the first labeled antigen binding molecule but not the second unlabeled antigen binding molecule. After incubation under conditions permissive for binding of the first antibody to the antigen, excess unbound antibody is removed, and the amount of label associated with immobilized antigen is measured. If the amount of label associated with immobilized antigen is substantially reduced in the test sample relative to the control sample, then that indicates that the second antigen binding molecule is competing with the first antigen binding molecule for binding to the antigen. See Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Trimeric antigen binding molecules of the invention prepared as described herein may be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography, size exclusion chromatography, and the like. The actual conditions used to purify a particular protein will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity etc., and will be apparent to those having skill in the art. For affinity chromatography purification an antibody, ligand, receptor or antigen can be used to which the trimeric antigen binding molecule binds. For example, for affinity chromatography purification of fusion proteins of the invention, a matrix with protein A or protein G may be used. Sequential Protein A or G affinity chromatography and size exclusion chromatography can be used to isolate an antigen binding molecule essentially as described in the Examples. The purity of the trimeric antigen binding molecule or fragments thereof can be determined by any of a variety of well-known analytical methods including gel electrophoresis, high pressure liquid chromatography, and the like. For example, the trimeric antigen binding molecule expressed as described in the Examples were shown to be intact and properly assembled as demonstrated by reducing and non-reducing SDS-PAGE.

The invention also encompasses a trimeric antigen binding molecule produced by the methods of the invention.

Assays

The trimeric antigen binding molecules provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

1. Affinity assays

The affinity of the trimeric antigen binding molecule provided herein for the corresponding TNF receptor can be determined in accordance with the methods set forth in the Examples by surface plasmon resonance (SPR), using standard instrumentation such as a BIAcore instrument (GE Healthcare), and receptors or target proteins such as may be obtained by recombinant expression. The affinity of the trimeric antigen binding molecule for the target cell antigen can also be determined by surface plasmon resonance (SPR), using standard instrumentation such as a BIAcore instrument (GE Healthcare), and receptors or target proteins such as may be obtained by recombinant expression. A specific illustrative and exemplary embodiment for measuring binding affinity is described in Example 4.1. According to one aspect, K_D is measured by surface plasmon resonance using a BIACORE® T200 machine (GE Healthcare) at 25 °C.

2. Binding assays and other assays

Binding of the trimeric antigen binding molecule provided herein to the corresponding receptor expressing cells may be evaluated using cell lines expressing the particular receptor or target antigen, for example by flow cytometry (FACS). In one aspect, fresh peripheral blood mononuclear cells (PBMCs) expressing the TNF receptor are used in the binding assay. These cells are used directly after isolation (naïve PMBCs) or after stimulation (activated PMBCs). In another aspect, activated mouse splenocytes (expressing the TNF receptor molecule) can be used to demonstrate binding of trimeric antigen binding molecule of the invention to the corresponding TNF receptor expressing cells.

In a further aspect, cancer cell lines expressing the target cell antigen, for example FAP, were used to demonstrate the binding of the antigen binding molecules to the target cell antigen.

In another aspect, competition assays may be used to identify an antigen binding molecule that competes with a specific antibody or antigen binding molecule for binding to the target or TNF receptor, respectively. In certain embodiments, such a competing antigen binding molecule binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by a specific anti-target antibody or a specific anti-TNF receptor antibody. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in *Methods in Molecular Biology* vol. 66 (Humana Press, Totowa, NJ).

3. Activity assays

In one aspect, assays are provided for identifying trimeric antigen binding molecules that bind to a specific target cell antigen and to a specific TNF receptor having biological activity. Biological activity may include, e.g., agonistic signalling through the TNF receptor on cells

expressing the TNF receptor. Trimeric antigen binding molecules identified by the assays as having such biological activity *in vitro* are also provided. In particular, a reporter cell assay detecting NF- κ B activation in Hela cells expressing human 4-1BB or human OX40 and co-cultured with FAP-expressing tumor cells is provided (see e.g. Example 5.1).

- 5 In certain aspects, trimeric antigen binding molecules of the invention are tested for such biological activity. Assays for detecting the biological activity of the molecules of the invention are those described in Example 5 or Example 8. In addition the biological activity of such complexes can be assessed by evaluating their effects on survival, proliferation and lymphokine secretion of various lymphocyte subsets such as NK cells, NKT-cells or $\gamma\delta$ T-cells or assessing
10 their capacity to modulate phenotype and function of antigen presenting cells such as dendritic cells, monocytes/macrophages or B-cells.

Pharmaceutical Compositions, Formulations and Routes of Administration

- In a further aspect, the invention provides pharmaceutical compositions comprising any of the trimeric antigen binding molecules provided herein, e.g., for use in any of the below
15 therapeutic methods. In one embodiment, a pharmaceutical composition comprises a trimeric antigen binding molecule and at least one pharmaceutically acceptable excipient. In another embodiment, a pharmaceutical composition comprises any of the trimeric antigen binding molecules provided herein and at least one additional therapeutic agent, e.g., as described below.

- Pharmaceutical compositions of the present invention comprise a therapeutically effective
20 amount of one or more trimeric antigen binding molecule dissolved or dispersed in a pharmaceutically acceptable excipient. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that are generally non-toxic to recipients at the dosages and concentrations employed, i.e. do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as
25 appropriate. The preparation of a pharmaceutical composition that contains at least one trimeric antigen binding molecule and optionally an additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. In particular, the compositions are lyophilized formulations or aqueous solutions. As
30 used herein, "pharmaceutically acceptable excipient" includes any and all solvents, buffers, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g. antibacterial agents, antifungal agents), isotonic agents, salts, stabilizers and combinations thereof, as would be known to one of ordinary skill in the art.

- Parenteral compositions include those designed for administration by injection, e.g.
35 subcutaneous, intradermal, intralesional, intravenous, intraarterial intramuscular, intrathecal or

intraperitoneal injection. For injection, the trimeric antigen binding molecules of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the fusion proteins may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. Sterile injectable solutions are prepared by incorporating the fusion proteins or trimeric antigen binding molecules of the invention in the required amount in the appropriate solvent with various of the other ingredients enumerated below, as required. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein. Suitable pharmaceutically acceptable excipients include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Aqueous injection suspensions may contain compounds which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, dextran, or the like. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleates or triglycerides, or liposomes.

Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (18th Ed. Mack Printing Company, 1990). Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, e.g. films, or microcapsules. In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

Exemplary pharmaceutically acceptable excipients herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US Patent No. 6,267,958. Aqueous antibody formulations include those described in US Patent No. 6,171,586 and WO2006/044908, the latter formulations including a histidine-acetate buffer.

In addition to the compositions described previously, the trimeric antigen binding molecules may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the trimeric antigen binding molecules may be formulated with suitable polymeric or hydrophobic materials (for example as emulsion in a pharmaceutically acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical compositions comprising the trimeric antigen binding molecules of the invention may be manufactured by means of conventional mixing, dissolving, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the proteins into preparations that

can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

The trimeric antigen binding molecules may be formulated into a composition in a free acid or base, neutral or salt form. Pharmaceutically acceptable salts are salts that substantially retain the biological activity of the free acid or base. These include the acid addition salts, e.g. those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

The pharmaceutical compositions may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended. In one aspect, the pharmaceutical composition comprises a trimeric antigen binding molecule and another active anti-cancer agent.

The formulations to be used for in vivo administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

Therapeutic methods and compositions

Any of the trimeric antigen binding molecules provided herein may be used in therapeutic methods. For use in therapeutic methods, the antigen binding molecules of the invention can be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

In one aspect, the trimeric antigen binding molecules of the invention are provided for use as a medicament. In further aspects, the trimeric antigen binding molecules of the invention are provided for use in treating a disease, in particular for use in the treatment of cancer. In certain embodiments, the trimeric costimulatory antigen binding molecules of the invention are provided for use in a method of treatment. In one embodiment, the invention provides a trimeric antigen binding molecule as described herein for use in the treatment of a disease in an individual in

need thereof. In certain embodiments, the invention provides a trimeric antigen binding molecule for use in a method of treating an individual having a disease comprising administering to the individual a therapeutically effective amount of the trimeric antigen binding molecule. In certain embodiments the disease to be treated is cancer. In certain embodiments the disease to be treated is a proliferative disorder, particularly cancer. Examples of cancers include bladder cancer, brain cancer, head and neck cancer, pancreatic cancer, lung cancer, breast cancer, ovarian cancer, uterine cancer, cervical cancer, endometrial cancer, esophageal cancer, colon cancer, colorectal cancer, rectal cancer, gastric cancer, prostate cancer, blood cancer, skin cancer, squamous cell carcinoma, bone cancer, and kidney cancer. Other cell proliferation disorders that can be treated using a trimeric antigen binding molecule of the present invention include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system. Also included are pre-cancerous conditions or lesions and cancer metastases. In certain embodiments the cancer is chosen from the group consisting of renal cell cancer, skin cancer, lung cancer, colorectal cancer, breast cancer, brain cancer, head and neck cancer. The subject, patient, or "individual" in need of treatment is typically a mammal, more specifically a human.

Also encompassed by the invention is the trimeric antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in up-regulating or prolonging cytotoxic T cell activity.

In a further aspect, the invention provides for the use of a trimeric antigen binding molecule of the invention in the manufacture or preparation of a medicament for the treatment of a disease in an individual in need thereof. In one aspect, the medicament is for use in a method of treating a disease comprising administering to an individual having the disease a therapeutically effective amount of the medicament. In certain embodiments the disease to be treated is a proliferative disorder, particularly cancer. Examples of cancers include bladder cancer, brain cancer, head and neck cancer, pancreatic cancer, lung cancer, breast cancer, ovarian cancer, uterine cancer, cervical cancer, endometrial cancer, esophageal cancer, colon cancer, colorectal cancer, rectal cancer, gastric cancer, prostate cancer, blood cancer, skin cancer, squamous cell carcinoma, bone cancer, and kidney cancer. Other cell proliferation disorders that can be treated using a trimeric antigen binding molecule of the present invention include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system. Also included are pre-cancerous conditions or lesions and cancer metastases. In certain embodiments the cancer is chosen from the group consisting of

renal cell cancer, skin cancer, lung cancer, colorectal cancer, breast cancer, brain cancer, head and neck cancer. A skilled artisan readily recognizes that in many cases the trimeric antigen binding molecule may not provide a cure but may only provide partial benefit. In some embodiments, a physiological change having some benefit is also considered therapeutically beneficial. Thus, in some embodiments, an amount of trimeric antigen binding molecule that provides a physiological change is considered an "effective amount" or a "therapeutically effective amount". In any of the above embodiments the individual is preferably a mammal, particularly a human.

In a further aspect, the invention provides a method for treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a trimeric antigen binding molecule of the invention. In one embodiment a composition is administered to said individual, comprising a fusion protein of the invention in a pharmaceutically acceptable form. In certain embodiments the disease to be treated is a proliferative disorder. In a particular embodiment the disease is cancer. In certain embodiments the method further comprises administering to the individual a therapeutically effective amount of at least one additional therapeutic agent, e.g. an anti-cancer agent if the disease to be treated is cancer. An "individual" according to any of the above embodiments may be a mammal, preferably a human.

For the prevention or treatment of disease, the appropriate dosage of a trimeric antigen binding molecule of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the route of administration, the body weight of the patient, the type of fusion protein, the severity and course of the disease, whether the fusion protein is administered for preventive or therapeutic purposes, previous or concurrent therapeutic interventions, the patient's clinical history and response to the fusion protein, and the discretion of the attending physician. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

The trimeric antigen binding molecule is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 $\mu\text{g/kg}$ to 15 mg/kg (e.g. 0.1 mg/kg – 10 mg/kg) of the antigen binding molecule can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 $\mu\text{g/kg}$ to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary

dosage of the fusion protein would be in the range from about 0.005 mg/kg to about 10 mg/kg. In other examples, a dose may also comprise from about 1 µg/kg body weight, about 5 µg/kg body weight, about 10 µg/kg body weight, about 50 µg/kg body weight, about 100 µg/kg body weight, about 200 µg/kg body weight, about 350 µg/kg body weight, about 500 µg/kg body weight, about 1 mg/kg body weight, about 5 mg/kg body weight, about 10 mg/kg body weight, about 50 mg/kg body weight, about 100 mg/kg body weight, about 200 mg/kg body weight, about 350 mg/kg body weight, about 500 mg/kg body weight, to about 1000 mg/kg body weight or more per administration, and any range derivable therein. In examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg body weight to about 100 mg/kg body weight, about 5 µg/kg body weight to about 500 mg/kg body weight etc., can be administered, based on the numbers described above. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the trimeric antigen binding molecule). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The trimeric antigen binding molecule of the invention will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent a disease condition, the trimeric antigen binding molecules of the invention, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays, such as cell culture assays. A dose can then be formulated in animal models to achieve a circulating concentration range that includes the IC₅₀ as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data, e.g., animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the trimeric antigen binding molecules which are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 50 mg/kg/day, typically from about 0.5 to 1 mg/kg/day. Therapeutically effective plasma levels may be achieved by

administering multiple doses each day. Levels in plasma may be measured, for example, by HPLC.

In cases of local administration or selective uptake, the effective local concentration of the trimeric antigen binding molecules may not be related to plasma concentration. One skilled in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

A therapeutically effective dose of the trimeric antigen binding molecules described herein will generally provide therapeutic benefit without causing substantial toxicity. Toxicity and therapeutic efficacy of a fusion protein can be determined by standard pharmaceutical procedures in cell culture or experimental animals. Cell culture assays and animal studies can be used to determine the LD₅₀ (the dose lethal to 50% of a population) and the ED₅₀ (the dose therapeutically effective in 50% of a population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which can be expressed as the ratio LD₅₀/ED₅₀. Trimeric antigen binding molecules that exhibit large therapeutic indices are preferred. In one embodiment, the trimeric antigen binding molecule according to the present invention exhibits a high therapeutic index. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosages suitable for use in humans. The dosage lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon a variety of factors, e.g., the dosage form employed, the route of administration utilized, the condition of the subject, and the like. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see, e.g., Fingl et al., 1975, in: The Pharmacological Basis of Therapeutics, Ch. 1, p. 1, incorporated herein by reference in its entirety).

The attending physician for patients treated with the trimeric antigen binding molecules of the invention will know how and when to terminate, interrupt, or adjust administration due to toxicity, organ dysfunction, and the like. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated, with the route of administration, and the like. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency will also vary according to the age, body weight, and response of the individual patient.

Other agents and treatments

The trimeric antigen binding molecules of the invention may be administered in combination with one or more other agents in therapy. For instance, a fusion protein of the

invention may be co-administered with at least one additional therapeutic agent. The term “therapeutic agent” encompasses any agent that can be administered for treating a symptom or disease in an individual in need of such treatment. Such additional therapeutic agent may comprise any active ingredients suitable for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. In certain embodiments, an additional therapeutic agent is another anti-cancer agent.

Such other agents are suitably present in combination in amounts that are effective for the purpose intended. The effective amount of such other agents depends on the amount of fusion protein used, the type of disorder or treatment, and other factors discussed above. The trimeric antigen binding molecules are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate compositions), and separate administration, in which case, administration of the trimeric antigen binding molecule of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant.

Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper that is pierceable by a hypodermic injection needle). At least one active agent in the composition is a trimeric antigen binding molecule of the invention.

The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises a trimeric antigen binding molecule of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition.

Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Table C. Sequences

SEQ ID NO:	Name	Sequence
1	Full length human CMP	MRVLSGTSLMLCSLLLLLQALCSPGLAPQSRGHLCTRPTDLVFVVDSSRSVRPVEFEKVKVFLSQVIESLDVGP NATRVGMVNYASTVKQEFSLRAHVSKAALLQAVRRIQPLSTGTMTGLAIQFAITKAFGDAEGGRSRS PDISKVVIVVTDGR PQDSVQDV SARARASGVELFAI GVGSVDKATLRQIASEPQDEHVDYVESYSVIEKLSR KFQEAFCVVSDLCATGDHDCEQVCISSPGSYTCAC HEGFTLNSDGKTCNVCSGGGGSSATDLVFLIDGSKS VRPENFELVKKFISQIVDTLDVSDKLAQVGLVQYSS SVRQEFPLGRFHTKKDIKAAVRNMSYMEKGTMTG AALKYLIDNSFTVSSGARPGAQKVGIVFTDGRSQDY INDAAKKAKDLGFKMFAVGVGNAVEDELREIASEP VAEHYFYTADFKTINQIGKKLQKKICVEEDPCACES LVKFQAKVEGLLQALTRKLEAVSKRLAILENTVV
2	CMP trimerization domain	CACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVV
3	Human OX40 ECD	Uniprot No. P43489, aa 29-214
4	OX40(8H9,49B4,1G4, 20B7) CDR-H1	SYAIS
5	OX40(CLC-563, CLC-564, 17A9) CDR-H1	SYAMS
6	OX40(8H9,49B4,1G4, 20B7) CDR-H2	GIIPFGTANYAQKFQG
7	OX40(CLC-563, CLC-564, 17A9) CDR-H2	AISGSGGSTYYADSVKG
8	OX40(8H9) CDR-H3	EYGWMDY
9	OX40(49B4) CDR-H3	EYYRGPYDY
10	OX40(1G4) CDR-H3	EYGSM DY
11	OX40(20B7) CDR-H3	VNYPYSYWGDFDY
12	OX40(CLC-563) CDR-H3	DVGAFDY
13	OX40(CLC-564) CDR-H3	DVGPF DY
14	OX40(17A9)-CDR-H3	VFYRGGVSM DY
15	OX40(8H9,49B4,1G4, 20B7) CDR-L1	RASQSISSWLA
16	OX40(CLC-563, CLC564) CDR-L1	RASQSVSSSYLA
17	OX40(17A9) CDR-L1	QGDSLRSYYAS
18	OX40(8H9,49B4,1G4, 20B7) CDR-L2	DASSLES

SEQ ID NO:	Name	Sequence
19	OX40(CLC-563, CLC564) CDR-L2	GASSRAT
20	OX40(17A9) CDR-L2	GKNNRPS
21	OX40(8H9) CDR-L3	QQYLTYSRFT
22	OX40(49B4) CDR-L3	QQYSSQPYT
23	OX40(1G4) CDR-L3	QQYISYSMLT
24	OX40(20B7) CDR-L3	QQYQAFSLT
25	OX40(CLC-563, CLC-164) CDR-L3	QQYGSSPLT
26	OX40(17A9) CDR-L3	NSRVMPHNRV
27	OX40(8H9) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIS WVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTI TADKSTSTAYMELSSLRSED TAVYYCAREY GWMD <u>YWGQGTTVTVSS</u>
28	OX40(8H9) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPDDFATYYCQQYLTYSRFTFGQG TKVEIK
29	OX40(49B4) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIS WVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTI TADKSTSTAYMELSSLRSED TAVYYCAREY YRGPY <u>DYWGQGTTVTVSS</u>
30	OX40(49B4) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPDDFATYYCQQYSSQPYTFGQG TKVEIK
31	OX40(1G4) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIS WVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTI TADKSTSTAYMELSSLRSED TAVYYCAREY GSMDY <u>WGQGTTVTVSS</u>
32	OX40(1G4) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPDDFATYYCQQYISYSMLTFGQG TKVEIK
33	OX40(20B7) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIS WVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTI TADKSTSTAYMELSSLRSED TAVYYCAR VNYPYSY <u>WGDFDYWGQGTTVTVSS</u>
34	OX40(20B7) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPDDFATYYCQQYQAFSLTFGQG TKVEIK
35	OX40(CLC-563) VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS WVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCALDVGAFD <u>YWGQGalVTVSS</u>
36	OX40(CLC-563) VL	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGSSPLTFGQG TKVEIK
37	OX40(CLC-564) VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS WVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAFDVGPFD <u>YWGQGTlVTVSS</u>
38	OX40(CLC-564) VL	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWY

SEQ ID NO:	Name	Sequence
		QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYGSSPLTFGQGTKVEIK
39	OX40(17A9) VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS WVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCARVFYRGG VSMDYWGQGTLLTVSS
40	OX40(17A9) VL	SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWY QQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTAS LTITGAQAEDEADYYCNSRVMPHNRVFGGGTKLTV
41	Human 4-1BB ECD	Uniprot No. Q07011, aa 24-186
42	4-1BB(12B3,11D5, 9B11, 20G2) CDR-H1	SYAIS
43	4-1BB(25G7) CDR-H1	SYAMS
44	4-1BB(12B3,11D5, 9B11, 20G2) CDR-H2	GIPIFGTANYAQKFQG
45	4-1BB(25G7) CDR-H2	AISGSGGSTYYADSVKG
46	4-1BB(12B3) CDR-H3	SEFRFYADFDY
47	4-1BB(25G7) CDR-H3	DDPWPPFDY
48	4-1BB(11D5) CDR-H3	STLIYGYFDY
49	4-1BB(9B11) CDR-H3	SSGAYPGYFDY
50	4-1BB(20G2) CDR-H3	SYWESYPFDY
51	4-1BB(12B3,11D5, 9B11, 20G2) CDR-L1	RASQSISSWLA
52	4-1BB(25G7) CDR-L1	QGDSLRSYYAS
53	4-1BB(12B3,11D5, 9B11, 20G2) CDR-L2	DASSLES
54	4-1BB(25G7) CDR-L2	GKNNRPS
55	4-1BB(12B3) CDR-L3	QQYHSYPQT
56	4-1BB(25G7) CDR-L3	NSLDRRGMWV
57	4-1BB(11D5) CDR-L3	QQLNSYPQT
58	4-1BB(9B11) CDR-L3	QQVNSYPQT
59	4-1BB(20G2) CDR-L3	QQQHSYYT
60	4-1BB(12B3) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAI WVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTI TADKSTSTAYMELSSLRSEDVAVYYCARSEFRFYA DFDYWGQGTLLTVSS
61	4-1BB(12B3) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPDDFATYYCQQYHSYPQTFGQGTKVEIK
62	4-1BB(25G7) VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS WVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCARDPWPP FDYWGQGTLLTVSS
63	4-1BB(25G7) VL	SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWY QQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTAS LTITGAQAEDEADYYCNSLDRRGMWVFGGGTKLTV
64	4-1BB(11D5) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAI WVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTI

SEQ ID NO:	Name	Sequence
		TADKSTSTAYMELSSLRSED TAVYYCAR <u>STLIYGYF</u> <u>DYWGQGTTVT</u> VSS
65	4-1BB(11D5) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPD DFATYYC <u>QQLNSYPQT</u> FGQGTKVEIK
66	4-1BB(9B11) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIS WVRQAPGQGLEWMGGIIPIFGTANYAOKFQGRVTI TADKSTSTAYMELSSLRSED TAVYYCAR <u>SSGAYPG</u> <u>YFDYWGQGTTVT</u> VSS
67	4-1BB(9B11) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPD DFATYYC <u>QQVNSYPQT</u> FGQGTKVEIK
68	4-1BB(20G2) VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIS WVRQAPGQGLEWMGGIIPIFGTANYAOKFQGRVTI TADKSTSTAYMELSSLRSED TAVYYCAR <u>SYYWESY</u> <u>PFDYWGQGTTVT</u> VSS
69	4-1BB(20G2) VL	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWY QQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTL TISSLQPD DFATYYC <u>QQQHSYYT</u> FGQGTKVEIK
70	FAP(28H1) CDR-H1	SHAMS
71	FAP(28H1) CDR-H2	AIWASGEQYYADSVKG
72	FAP(28H1) CDR-H3	GWLG NFDY
73	FAP(28H1) CDR-L1	RASQSVRSYLA
74	FAP(28H1) CDR-L2	GASTRAT
75	FAP(28H1) CDR-L3	QQGQVIPPT
76	FAP(4B9) CDR-H1	SYAMS
77	FAP(4B9) CDR-H2	AIIGSGASTYYADSVKG
78	FAP(4B9) CDR-H3	GWFGGFNY
79	FAP(4B9) CDR-L1	RASQSVTSSYLA
80	FAP(4B9) CDR-L2	VGSRRAT
81	FAP(4B9) CDR-L3	QQGIMLPPT
82	FAP(28H1) VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSHAMS WVRQAPGKGLEWVSAIWASGEQYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAKGWLG NF <u>DYWGQGT</u> LVTVSS
83	FAP(28H1) VL	EIVLTQSPGTLSLSPGERATLSCRASQSVRSYLA WYQQKPGQAPRLLIIGASTRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVYYCQQGQVIPPTFGQGTKVEIK
84	FAP(4B9) VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS WVRQAPGKGLEWVSAIIGSGASTYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAKGWFGGF NYWGQGTLVTVSS
85	FAP(4B9) VL	EIVLTQSPGTLSLSPGERATLSCRASQSVTSSYLA WYQQKPGQAPRLINVGSRRTGIPDRFSGSGSGTDFT LTISRLEPEDFAVYYCQQGIMLPPTFGQGTKVEIK
86	Human (hu) FAP	UniProt no. Q12884
87	hu FAP ectodomain+poly-lys-tag+his ₆ -tag	RPSRVHNSEENTMRALTLKDILNGTFSYKTFFPNWI SGQEYLHQ SADNNIVLYNIETGQSYTILSNRTMKS VNASNYGLSPDRQFVYLESDY SKLWRY SYTATYYIY DLSNGEFVRG NELPRPIQYLCWSPVGS KLAYVYQN

SEQ ID NO:	Name	Sequence
		NIYLKQRPDPPFQITFNGRENKIFNGIPDWVYEEE MLATKYALWWSPNGKFLAYAEFNDTDIPVIAYSYY GDEQYPR TINIPYPKAGAKNPVVRIFIIDTTYPAYVG PQEVPVPAMIASSDYYFSWLTWVTDERVCLQWLK RVQNVSVLSICDFREDWQWDCPKTQEHIEESRTG WAGGFFVSTPVFSYDAISYYKIFSDKDGYKHIHYIK DTVENAIQITSGKWEAINIFRVTQDSLFISSNEFEEY PGRRNIRISIGSYPPSKKCVTCHLRKERCQYYTASF SDYAKYYALVCYGPPISTLHDGR TDQEI KILEENK ELENALKNIQLPKKEIKKLEVDEITLWYKMILPPQFD RSKKYPLLIQVYGGPCSQSVRSVFAVNWISYLASKE GMVIALVDGRGTAFQGDKLLYAVYRKLGVYEVED QITAVRKFIEMGFIDEKRIAIWGW SYGGYVSSLALA SGTGLFKCGIAVAPVSSWEYYASVYTERFMGLPTK DDNLEHYKNSTVMARA EYFRNVDYLLIHGTADDN VHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSG LSTNHL YTHMTHFLKQCFSLS DGKKKKKKKGHHHH HH
88	nucleotide sequence hu FAP ectodomain+poly-lys- tag+his ₆ -tag	CGCCCTTCAAGAGTTCATAACTCTGAAGAAAATA CAATGAGAGCACTCACACTGAAGGATATTTTAAA TGGAACATTTTCTTATAAAAACATTTTTCCAA ACTGGATTT CAGGACAAGAATATCTTCATCAATCTGC AGATAACAATATAGTACTTTATAATATTGAAACA GGACAATCATATAACCATTTTGAGTAATAGAACCA TGAAAAGTGTGAATGCTTCAAATTACGGCTTATC ACCTGATCGGCAATTTGTATATCTAGAAAGTGAT TATTCAAAGCTTTGGAGATACTCTTACACAGCAA CATATTACATCTATGACCTTAGCAATGGAGAATT TGTAAGAGGAAATGAGCTTCCTCGTCCAATTCAG TATTTATGCTGGTCGCCTGTTGGGAGTAAATTAG CATATGTCTATCAAAACAATATCTATTTGAAACA AAGACCAGGAGATCCACCTTTTCAAATAACATTT AATGGAAGAGAAAATAAAATATTTAATGGAATC CCAGACTGGGTTTATGAAGAGGAAATGCTTGCTA CAAAATATGCTCTCTGGTGGTCTCCTAATGGAAA ATTTTGGCATATGCGGAATTTAATGATACGGAT ATACCAGTTATTGCCTATTCCTATTATGGCGATGA ACAATATCCTAGAACAAATAAATATTCCATACCCA AAGGCTGGAGCTAAGAATCCCGTTGTTCCGATAT TTATTATCGATAACCACTTACCCTGCGTATGTAGGT CCCCAGGAAGTGCCTGTTCCAGCAATGATAGCCT CAAGTGATTATTATTTTCA GTTGGCTCACGTGGGTT ACTGATGAACGAGTATGTTTGCAGTGGCTAAAAA GAGTCCAGAATGTTTCGGTCCTGTCTATATGTGA CTT CAGGGAAGACTGGCAGACATGGGATTGTCCA AAGACCCAGGAGCATATAGAAGAAAGCAGAACT GGATGGGCTGGTGGATTCTTTGTTTCAACACCAG TTTTCAGCTATGATGCCATTTCTGACTACAAAATA TTTAGTGACAAGGATGGCTACAAACATATTCACT ATATCAAAGACACTGTGGAAAATGCTATTCAAAT TACAAGTGGCAAGTGGGAGGCCATAAATATATTC AGAGTAACACAGGATTCACTGTTTTATTCTAGCA

SEQ ID NO:	Name	Sequence
		ATGAATTTGAAGAATACCCTGGAAGAAGAAACA TCTACAGAATTAGCATTGGAAGCTATCCTCCAAG CAAGAAGTGTGTTACTTGCCATCTAAGGAAAGAA AGGTGCCAATATTACACAGCAAGTTTCAGCGACT ACGCCAAGTACTATGCACTTGTCTGCTACGGCCC AGGCATCCCCATTTCCACCCTTCATGATGGACGC ACTGATCAAGAAATTAATAATCCTGGAAGAAAAC AAGGAATTGGAAAATGCTTTGAAAAATATCCAGC TGCCTAAAGAGGAAATTAAGAACTTGAAGTAG ATGAAATTACTTTATGGTACAAGATGATTCTTCCT CCTCAATTTGACAGATCAAAGAAGTATCCCTTGC TAATTCAAGTGTATGGTGGTCCCTGCAGTCAGAG TGTAAGGTCTGTATTTGCTGTTAATTGGATATCTT ATCTTGCAAGTAAGGAAGGGATGGTCATTGCCTT GGTGGATGGTCGAGGAACAGCTTTCCAAGGTGAC AAACTCCTCTATGCAGTGTATCGAAAGCTGGGTG TTTATGAAGTTGAAGACCAGATTACAGCTGTCAG AAAATTCATAGAAATGGGTTTCATTGATGAAAAA AGAATAGCCATATGGGGCTGGTCCTATGGAGGAT ACGTTTCATCACTGGCCCTTGCATCTGGAAGTGGT CTTTTCAAATGTGGTATAGCAGTGGCTCCAGTCTC CAGCTGGGAATATTACGCGTCTGTCTACACAGAG AGATTCATGGGTCTCCCAACAAAGGATGATAATC TTGAGCACTATAAGAATTCAACTGTGATGGCAAG AGCAGAATATTTAGAAATGTAGACTATCTTCTC ATCCACGGAACAGCAGATGATAATGTGCACTTTC AAAACCTCAGCACAGATTGCTAAAGCTCTGGTTAA TGCACAAGTGGATTTCCAGGCAATGTGGTACTCT GACCAGAACCACGGCTTATCCGGCCTGTCCACGA ACCACTTATACACCCACATGACCCACTTCCTAAA GCAGTGTTTCTCTTTGTCAGACGGCAAAAAGAAA AAGAAAAAGGGCCACCACCATCACCATCAC
89	mouse FAP	UniProt no. P97321
90	Murine FAP ectodomain+poly-lys- tag+his ₆ -tag	RPSRVYKPEGNTKRALTLKDILNGTFSYKTYFPNWI SEQEYLHQSEDDNIVFYNIETRESYIILSNSTMKSVN ATDYGLSPDRQFVYLESDYSKLWRYSYTATYYIYD LQNGEFVRGYELPRPIQYLCWSPVGSKLAYVYQNN IYLKQRPDPPFQITYTGRENRIENGIPDWVYEEEML ATKYALWWSPDGKFLAYVEFNDSDIPIIAYSYYGD GQYPRTINIPYPKAGAKNPVVRVFIVDTTYPHHVGP MEVPVPEMIASSDYYFSWLTWVSSERVCLQWLKR VQNVSVLSICDFREDWHAWECPKNQEHEVESRTG WAGGFFVSTPAFSQDATSYKIFSDKDGYKHIHIK DTVENAIQITSGKWEAIYIFRVTQDSLFISSNEFEGY PGRRNIRISIGNSPPSKKCVTCHLRKERCQYYTASF SYKAKYYALVCYGPGLPISTLHDGRDQEIQVLEEN KELENSLRNIQLPKVEIKKLKDGGTLFWYKMILPPQ FDRSKKYPLLIQVYGGPCSQSVKSVFAVNWITYLAS KEGIVIALVDGRGTAFAQGDKFLHAVYRKLGVYEVE DQLTAVRKFIEMGFIDEERIAIWGWSYGGYVSSLAL ASGTGLFKCGIAVAPVSSWEYYASIYSEFMGLPTK DDNLEHYKNSTVMARAEYFRNVLDYLLIHGTADDN

SEQ ID NO:	Name	Sequence
		VHFQNSAQIAKALVNAQVDFQAMWYSDQNHGILS GRSQNHLYTHMTHFLKQCFSLSDGKKKKKKKGHHH HHH
91	nucleotide sequence of murine FAP ectodomain+poly-lys- tag+his ₆ -tag	CGTCCCTCAAGAGTTTACAAACCTGAAGGAAACA CAAAGAGAGCTCTTACCTTGAAGGATATTTTAAA TGGAACATTCTCATATAAAACATATTTTCCCAACT GGATTTTCAGAACAAGAATATCTTCATCAATCTGA GGATGATAACATAGTATTTTATAATATTGAAACA AGAGAATCATATATCATTTTGAGTAATAGCACCA TGAAAAGTGTGAATGCTACAGATTATGGTTTGTG ACCTGATCGGCAATTTGTGTATCTAGAAAGTGAT TATTCAAAGCTCTGGCGATATTCATACACAGCGA CATACTACATCTACGACCTTCAGAATGGGGAATT TGTAAGAGGATACGAGCTCCCTCGTCCAATTCAG TATCTATGCTGGTCGCCTGTTGGGAGTAAATTAG CATATGTATATCAAAACAATATTTATTTGAAACA AAGACCAGGAGATCCACCTTTTCAAATAACTTAT ACTGGAAGAGAAAATAGAATATTTAATGGAATA CCAGACTGGGTTTATGAAGAGGAAATGCTTGCCA CAAAATATGCTCTTTGGTGGTCTCCAGATGGAAA ATTTTTGGCATATGTAGAATTTAATGATTGAGATA TACCAATTATTGCCTATTCTTATTATGGTGATGGA CAGTATCCTAGAACTATAAATATTCCATATCCAA AGGCTGGGGCTAAGAATCCGGTTGTTCTGTGTTTT TATTGTTGACACCACCTACCCTCACACGTGGGC CCAATGGAAGTGCCAGTTCCAGAAATGATAGCCT CAAGTGACTATTATTTTCACTGGCTCACATGGGT GTCCAGTGAACGAGTATGCTTGCAGTGGCTAAAA AGAGTGCAGAATGTCTCAGTCCTGTCTATATGTG ATTTCAAGGAAGACTGGCATGCATGGGAATGTCC AAAGAACCAGGAGCATGTAGAAGAAAGCAGAAC AGGATGGGCTGGTGGATTCTTTGTTTCGACACCA GCTTTTAGCCAGGATGCCACTTCTTACTACAAAA TATTTAGCGACAAGGATGGTTACAAACATATTCA CTACATCAAAGACACTGTGGAATAATGCTATTCAA ATTACAAGTGGCAAGTGGGAGGCCATATATATAT TCCGCGTAACACAGGATTCACTGTTTTATTCTAGC AATGAATTTGAAGGTTACCCTGGAAGAAGAAAC ATCTACAGAATTAGCATTGGAAACTCTCCTCCGA GCAAGAAGTGTGTTACTTGCCATCTAAGGAAAGA AAGGTGCCAATATTACACAGCAAGTTTCAGCTAC AAAGCCAAGTACTATGCACTCGTCTGCTATGGCC CTGGCCTCCCCATTTCCACCCTCCATGATGGCCG ACAGACCAAGAAATACAAGTATTAGAAGAAAC AAAGAACTGGAAAATTCTCTGAGAAATATCCAGC TGCCTAAAGTGGAGATTAAGAAGCTCAAAGACG GGGACTGACTTTCTGGTACAAGATGATTCTGCC TCCTCAGTTTGACAGATCAAAGAAGTACCCTTTG CTAATTCAAGTGTATGGTGGTCCTTGTAGCCAGA GTGTTAAGTCTGTGTTTGTGTTAATTGGATAACT TATCTCGCAAGTAAGGAGGGGATAGTCATTGCCC TGGTAGATGGTCGGGGCACTGCTTTCCAAGGTGA

SEQ ID NO:	Name	Sequence
		CAAATTCCTGCATGCCGTGTATCGAAAACCTGGGT GTATATGAAGTTGAGGACCAGCTCACAGCTGTCA GAAAATTCATAGAAATGGGTTTCATTGATGAAGA AAGAATAGCCATATGGGGCTGGTCCTACGGAGGT TATGTTTCATCCCTGGCCCTTGCATCTGGAACCTGG TCTTTTCAAATGTGGCATAGCAGTGGCTCCAGTCT CCAGCTGGGAATATTACGCATCTATCTACTCAGA GAGATTCATGGGCCTCCCAACAAAGGACGACAAT CTCGAACACTATAAAAATTCAACTGTGATGGCAA GAGCAGAATATTTTCAGAAATGTAGACTATCTTCT CATCCACGGAACAGCAGATGATAATGTGCACTTT CAGAACTCAGCACAGATTGCTAAAGCTTTGGTTA ATGCACAAGTGGATTTCAGGCGATGTGGTACTC TGACCAGAACCATGGTATATTATCTGGGCGCTCC CAGAATCATTTATATACCCACATGACGCACTTCC TCAAGCAATGCTTTTCTTTATCAGACGGCAAAAA GAAAAAGAAAAAGGGCCACCACCATCACCATCA C
92	Cynomolgus FAP ectodomain+poly-lys- tag+his ₆ -tag	RPPRVHNSEENTMRALTLKDILNGTFSYKTFFPNWI SGQEYLHQSDADNNIVLYNIETGQSYTILSNRTMKSV NASNYGLSPDRQFVYLESDYSKLWRYSYTATYYIY DLSNGEFVRGNELPRPIQYLCWSPVGSKLAYVYQN NIYLKQRPQDPPFQITFNGRENKIFNGIPDWYEEE MLATKYALWWSPNGKFLAYAEFNDTDIPVIAYSYY GDEQYPRITINIPYPKAGAKNPFVRIFIIDTTYPAYVG PQEVVPAMIASSDYYFSWLTWVTDERVCLQWLK RVQNVSVLSICDFREDWQTWDCPKTQEHIEESRTG WAGGFFVSTPVFSYDAISYYKIFSDKDGKXHHYIK DTVENAIQITSGKWEAINIFRVTQDSLFISSNEFEDY PGRRNIRISIGSYPPSKKCVTCHLRKERCQYYTASF SDYAKYYALVCYGPPISTLHDGRDQEIKILEENK ELENALKNIQLPKEEIKKLEVDEITLWYKMILPPQFD RSKKYPLLIQVYGGPCSQSVRSVFVNWISYLASKE GMVIALVDGRGTAFQGDKLLYAVYRKLGVYEVED QITAVRKFIEMGFIDEKRIAIWGWSSYGGYVSSLALA SGTGLFKCGIAVAPVSSWEYYASVYTERFMGLPTK DDNLEHYKNSTVMARAEYFRNVYLLIHGTADDN VHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSG LSTNHLYTHMTHFLKQCFSLSDGKKKKKKGHHHH HH
93	nucleotide sequence of cynomolgus FAP ectodomain+poly-lys- tag+his ₆ -tag	CGCCCTCCAAGAGTTCATAACTCTGAAGAAAATA CAATGAGAGCACTCACACTGAAGGATATTTTAAA TGGGACATTTTCTTATAAAACATTTTTCCAAACCT GGATTTCAAGGACAAGAATATCTTCATCAATCTGC AGATAACAATATAGTACTTTATAATATTGAAACA GGACAATCATATACCATTTTGAGTAACAGAACCA TGAAAAGTGTGAATGCTTCAAATTATGGCTTATC ACCTGATCGGCAATTTGTATATCTAGAAAGTGAT TATTCAAAGCTTTGGAGATACTCTTACACAGCAA CATATTACATCTATGACCTTAGCAATGGAGAATT TGTAAGAGGAAATGAGCTTCCTCGTCCAATTCAG TATTTATGCTGGTCGCCTGTTGGGAGTAAATTAG

SEQ ID NO:	Name	Sequence
		CATATGTCTATCAAAACAATATCTATTTGAAACA AAGACCAGGAGATCCACCTTTTCAAATAACATTT AATGGAAGAGAAAATAAAATATTTAATGGAATC CCAGACTGGGTTTATGAAGAGGAAATGCTTGCTA CAAAATATGCTCTCTGGTGGTCTCCTAATGGAAA ATTTTTGGCATATGCGGAATTTAATGATACAGAT ATACCAGTTATTGCCTATTCCCTATTATGGCGATGA ACAATATCCCAGAACAATAAATATTCCATACCCA AAGGCCGGAGCTAAGAATCCTTTTGTTCCGATAT TTATTATCGATACCACTTACCCTGCGTATGTAGGT CCCCAGGAAGTGCCTGTTCCAGCAATGATAGCCT CAAGTGATTATTATTTCAAGTTGGCTCACGTGGGTT ACTGATGAACGAGTATGTTTGCAGTGGCTAAAAA GAGTCCAGAATGTTTCGGTCTTGTCTATATGTGAT TTCAGGGAAGACTGGCAGACATGGGATTGTCCAA AGACCCAGGAGCATATAGAAGAAAGCAGAACTG GATGGGCTGGTGGATTCTTTGTTTCAACACCAGTT TTCAGCTATGATGCCATTTCACTACTACAAAATATT TAGTGACAAGGATGGCTACAAACATATTTCACTAT ATCAAAGACACTGTGGAAAATGCTATTCAAATTA CAAGTGGCAAGTGGGAGGCCATAAATATATTTCAG AGTAACACAGGATTTCACTGTTTTATTCTAGCAAT GAATTTGAAGATTACCCTGGAAGAAGAAACATCT ACAGAATTAGCATTGGAAGCTATCCTCCAAGCAA GAAGTGTGTTACTTGCCATCTAAGGAAAGAAAGG TGCCAATATTACACAGCAAGTTTCAGCGACTACG CCAAGTACTATGCACTTGTCTGCTATGGCCCAGG CATCCCCATTTCCACCCTTCATGACGGACGCACT GATCAAGAAATTAATAATCCTGGAAGAAAACAAG GAATTGGAAAATGCTTTGAAAAATATCCAGCTGC CTAAAGAGGAAATTAAGAACTTGAAGTAGATG AAATTACTTTATGGTACAAGATGATTCTTCCTCCT CAATTTGACAGATCAAAGAAGTATCCCTTGCTAA TTCAAGTGTATGGTGGTCCCTGCAGTCAGAGTGT AAGGTCTGTATTTGCTGTTAATTGGATATCTTATC TTGCAAGTAAGGAAGGGATGGTCATTGCCTTGGT GGATGGTCTGGGGAACAGCTTTCCAAGGTGACAA ACTCCTGTATGCAGTGTATCGAAAGCTGGGTGTT TATGAAGTTGAAGACCAGATTACAGCTGTCAGAA AATTCATAGAAATGGGTTTCATTGATGAAAAAAG AATAGCCATATGGGGCTGGTCCTATGGAGGATAT GTTTCATCACTGGCCCTTGCATCTGGAAGTGGTCT TTTCAAATGTGGGATAGCAGTGGCTCCAGTCTCC AGCTGGGAATATTACGCGTCTGTCTACACAGAGA GATTCATGGGTCTCCCAACAAAGGATGATAATCT TGAGCACTATAAGAATTCAACTGTGATGGCAAGA GCAGAATATTTAGAAATGTAGACTATCTTCTCA TCCACGGAACAGCAGATGATAATGTGCACTTTCA AACTCAGCACAGATTGCTAAAGCTCTGGTTAAT GCACAAGTGGATTTCCAGGCAATGTGGTACTCTG ACCAGAACCACGGCTTATCCGGCCTGTCCACGAA CCACTTATACACCCACATGACCCACTTCCTAAAG

SEQ ID NO:	Name	Sequence
		CAGTGTCTTCTCTTTGTCAGACGGCAAAAAGAAAAAGAAAAAGGGCCACCACCATCACCATCAC
94	human CEA	UniProt no. P06731
95	human MCSP	UniProt no. Q6UVK1
96	human EGFR	UniProt no. P00533
97	human CD19	UniProt no. P15391
98	human CD20	Uniprot no. P11836
99	human CD33	UniProt no. P20138
100	human OX40	UniProt no. P43489
101	human 4-1BB	UniProt no. Q07011
102	human CD27	UniProt no. P26842
103	human HVEM	UniProt no. Q92956
104	human CD30	UniProt no. P28908
105	human GITR	UniProt no. Q9Y5U5
106	murine OX40	UniProt no. P47741
107	murine 4-1BB	UniProt no. P20334
108	cynomolgus 4-1BB	Uniprot no. F6W5G6
109	Peptide linker G4S	GGGGS
110	Peptide linker (G4S) ₂	GGGGS GGGGS
111	Peptide linker (SG4) ₂	SGGGSGGGG
112	Peptide linker (G ₄ S) ₃	GGGSGGGSGGGGS
113	Peptide linker G4(SG4) ₂	GGGSGGGSGGGG
114	Peptide linker (G ₄ S) ₄	GGGSGGGSGGGSGGGGS
115	Peptide linker	GSPGSSSSGS
116	Peptide linker	GSGSGSGS
117	Peptide linker	GSGSGNGS
118	Peptide linker	GGSGSGSG
119	Peptide linker	GGSGSG
120	Peptide linker	GGSG
121	Peptide linker	GGSGNGSG
122	Peptide linker	GGNGSGSG
123	Peptide linker	GGNGSG
124	human TRAF2	UniProt no. Q12933
125	human Thrombospondin 1	UniProt no. P07996
126	human Matrilin-4	UniProt no. O95460
127	human Cubilin	UniProt no. O60494
128	isoleucine zipper domain	IKQIEDKIEE ILSKIYHIEN EIARIKKLIG ER
129	cynomolgus OX40 ECD	aa 29-214
130	murine OX40 ECD	aa 10-211
131	nucleotide sequence Fc hole chain	see Table 2
132	nucleotide sequence human OX40 antigen Fc knob chain	see Table 2
133	nucleotide sequence	see Table 2

SEQ ID NO:	Name	Sequence
	cynomolgus OX40 antigen Fc knob chain	
134	nucleotide sequence murine OX40 antigen Fc knob chain	see Table 2
135	Fc hole chain	see Table 2
136	human OX40 antigen Fc knob chain	see Table 2
137	cynomolgus OX40 antigen Fc knob chain	see Table 2
138	murine OX40 antigen Fc knob chain	see Table 2
139	nucleotide sequence of library DP88-4	see Table 3
140	nucleotide sequence of Fab light chain Vk1_5	see Table 4
141	Fab light chain Vk1_5	see Table 4
142	nucleotide sequence of Fab heavy chain VH1_69	see Table 4
143	Fab heavy chain VH1_69	see Table 4
144	LMB3	see Table 5
145	Vk1_5_L3r_S	see Table 5
146	Vk1_5_L3r_SY	see Table 5
147	Vk1_5_L3r_SPY	see Table 5
148	RJH31	see Table 5
149	RJH32	see Table 5
150	DP88-v4-4	see Table 5
151	DP88-v4-6	see Table 5
152	DP88-v4-8	see Table 5
153	fdseqlong	see Table 5
154	(Vk3_20/VH3_23) template	see Table 6
155	nucleotide sequence of Fab light chain Vk3_20	see Table 7
156	Fab light chain Vk3_20	see Table 7
157	nucleotide sequence of Fab heavy chain VH3_23	see Table 7
158	Fab heavy chain VH3_23 (DP47)	see Table 7
159	MS64	see Table 8
160	DP47CDR3_ba (mod.)	see Table 8

SEQ ID NO:	Name	Sequence
161	DP47-v4-4	see Table 8
162	DP47-v4-6	see Table 8
163	DP47-v4-8	see Table 8
164	fdseqlong	see Table 8
165	VL3_19/VH3_23 library template	see Table 9
166	nucleotide sequence of Fab light chain VL3_19	see Table 10
167	Fab light chain VL3_19	see Table 10
168	LMB3	see Table 11
169	VL_3_19_L3r_V	see Table 11
170	VL_3_19_L3r_HV	see Table 11
171	VL_3_19_L3r_HLV	see Table 11
172	RJH80	see Table 11
173	Nucleotide sequence OX40(8H9) VL	see Table 12
174	Nucleotide sequence OX40(8H9) VH	see Table 12
175	Nucleotide sequence OX40(49B4) VL	see Table 12
176	Nucleotide sequence OX40(49B4) VH	see Table 12
177	Nucleotide sequence OX40(1G4) VL	see Table 12
178	Nucleotide sequence OX40(1G4) VH	see Table 12
179	Nucleotide sequence OX40(20B7) VL	see Table 12
180	Nucleotide sequence OX40(20B7) VH	see Table 12
181	Nucleotide sequence OX40(CLC-563) VL	see Table 12
182	Nucleotide sequence OX40(CLC-563) VH	see Table 12
183	Nucleotide sequence OX40(CLC-564) VL	see Table 12
184	Nucleotide sequence OX40(CLC-564) VH	see Table 12
185	Nucleotide sequence OX40(17A9) VL	see Table 12
186	Nucleotide sequence OX40(17A9) VH	see Table 12

SEQ ID NO:	Name	Sequence
187	cynomolgus 4-1BB ECD	aa 24-186
188	murine 4-1BB ECD	P20334, aa 24-187
189	human 4-1BB antigen Fc knob chain	nucleotide sequence, see Table 14
190	cynomolgus 4-1BB antigen Fc knob chain	nucleotide sequence, see Table 14
191	murine 4-1BB antigen Fc knob chain	nucleotide sequence, see Table 14
192	human 4-1BB antigen Fc knob chain	see Table 14
193	cynomolgus 4-1BB antigen Fc knob chain	see Table 14
194	murine 4-1BB antigen Fc knob chain	see Table 14
195	Primer MS63	see Table 11
196	Nucleotide sequence 4-1BB(12B3) VL	see Table 15
197	Nucleotide sequence 4-1BB(12B3) VH	see Table 15
198	Nucleotide sequence 4-1BB(25G7) VL	see Table 15
199	Nucleotide sequence 4-1BB(25G7) VH	see Table 15
200	Nucleotide sequence 4-1BB(11D5) VL	see Table 15
201	Nucleotide sequence 4-1BB(11D5) VH	see Table 15
202	Nucleotide sequence 4-1BB(9B11) VL	see Table 15
203	Nucleotide sequence 4-1BB(9B11) VH	see Table 15
204	Nucleotide sequence 4-1BB(20G2) VL	see Table 15
205	Nucleotide sequence 4-1BB(20G2) VH	see Table 15
206	12B3 P329GLALA IgG1 (light chain)	nucleotide sequence, see Table 16
207	12B3 P329GLALA IgG1 (heavy chain)	nucleotide sequence, see Table 16
208	12B3 P329GLALA IgG1 (light chain)	see Table 16
209	12B3 P329GLALA IgG1 (heavy chain)	see Table 16
210	25G7 P329GLALA IgG1 (light chain)	nucleotide sequence, see Table 16

SEQ ID NO:	Name	Sequence
211	25G7 P329GLALA IgG1 (heavy chain)	nucleotide sequence, see Table 16
212	25G7 P329GLALA IgG1 (light chain)	see Table 16
213	25G7 P329GLALA IgG1 (heavy chain)	see Table 16
214	3+3 (8H9/28H1) Light chain (LC) (pCON184)	nucleotide sequence, see Table 18
215	3+3 (8H9/28H1) CMP chain pETR12239	nucleotide sequence, see Table 18
216	3+3 (1G4/28H1) LC (pCON251)	nucleotide sequence, see Table 18
217	3+3 (1G4/28H1)CMP chain (pETR12640)	nucleotide sequence, see Table 18
218	3+3 (49B4/28H1) LC (pCON323)	nucleotide sequence, see Table 18
219	3+3 (49B4/28H1) CMP chain (pETR12899)	nucleotide sequence, see Table 18
220	3+3 (21H4/28H1) LC (pETR12026)	nucleotide sequence, see Table 18
221	3+3 (21H4/28H1) CMP chain (pETR12810)	nucleotide sequence, see Table 18
222	3+3 (CLC563/28H1) LC (pCON586)	nucleotide sequence, see Table 18
223	3+3 (CLC563/28H1) CMP chain (pETR12898)	nucleotide sequence, see Table 18
224	3+3 (20B7/28H1) LC (pCON260)	nucleotide sequence, see Table 18
225	3+3 (20B7/28H1) CMP chain (pETR12639)	nucleotide sequence, see Table 18
226	3+3 (8H9/28H1) Light chain (LC) (pCON184)	see Table 19
227	3+3 (8H9/28H1) CMP chain pETR12239	see Table 19
228	3+3 (1G4/28H1) LC (pCON251)	see Table 19
229	3+3 (1G4/28H1)CMP chain (pETR12640)	see Table 19
230	3+3 (49B4/28H1) LC (pCON323)	see Table 19
231	3+3 (49B4/28H1) CMP chain (pETR12899)	see Table 19
232	3+3 (21H4/28H1) LC (pETR12026)	see Table 19
233	3+3 (21H4/28H1) CMP chain (pETR12810)	see Table 19

SEQ ID NO:	Name	Sequence
234	3+3 (CLC563/28H1) LC (pCON586)	see Table 19
235	3+3 (CLC563/28H1) CMP chain (pETR12898)	see Table 19
236	3+3 (20B7/28H1) LC (pCON260)	see Table 19
237	3+3 (20B7/28H1) CMP chain (pETR12639)	see Table 19
238	12B3 VHCH1(4-1BB)-CMPtd-scFv (28H1)	nucleotide sequence, see Table 25
239	12B3 VLCL-Light chain 1 (4-1BB)	nucleotide sequence, see Table 25
240	25G7 VHCH1(4-1BB)-CMPtd-scFv (28H1)	nucleotide sequence, see Table 25
241	25G7 VLCL-Light chain 1 (4-1BB)	nucleotide sequence, see Table 25
242	11D5 VHCH1(4-1BB)-CMPtd-scFv (28H1)	nucleotide sequence, see Table 25
243	11D5 VLCL-Light chain 1 (4-1BB)	nucleotide sequence, see Table 25
244	9B11 VHCH1(4-1BB)-CMPtd-scFv (28H1)	nucleotide sequence, see Table 25
245	9B11 VLCL-Light chain 1 (4-1BB)	nucleotide sequence, see Table 25
246	12B3 VHCH1(4-1BB)-CMPtd-scFv (28H1)	see Table 26
247	12B3 VLCL-Light chain 1 (4-1BB)	see Table 26
248	25G7 VHCH1(4-1BB)-CMPtd-scFv (28H1)	see Table 26
249	25G7 VLCL-Light chain 1 (4-1BB)	see Table 26
250	11D5 VHCH1(4-1BB)-CMPtd-scFv (28H1)	see Table 26
251	11D5 VLCL-Light chain 1 (4-1BB)	see Table 26
252	9B11 VHCH1(4-1BB)-CMPtd-scFv (28H1)	see Table 26
253	9B11 VLCL-Light chain 1 (4-1BB)	see Table 26

The following points describe aspects of the present invention:

- (1) A trimeric antigen binding molecule comprising three fusion polypeptides, each of the three fusion polypeptides comprising

- (a) a moiety capable of specific binding to a costimulatory TNF receptor family member,
- (b) a trimerization domain, and
- (c) a moiety capable of specific binding to a target cell antigen.

(2) The trimeric antigen binding molecule as defined in the point (1) above, comprising three fusion polypeptides, each of the three fusion polypeptides comprising

- (a) a moiety capable of specific binding to a costimulatory TNF receptor family member,
- (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1, and
- (c) a moiety capable of specific binding to a target cell antigen.

(3) The trimeric antigen binding molecule as defined in point (1) or point (2) above, wherein the trimerization domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO:2.

(4) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the trimerization domain comprises the amino acid sequence of SEQ ID NO:2.

(5) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the three fusion polypeptides are linked by disulphide bonds.

(6) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the costimulatory TNF receptor family member is selected from OX40 and 4-1BB.

(7) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:3.

(8) The trimeric antigen binding molecule as defined in any one of the preceding points, comprising a moiety capable of specific binding to OX40, wherein the moiety comprises a VH comprising

- (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5,
- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and
- (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14,

and a VL comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25 and SEQ ID NO:26.

(9) The trimeric antigen binding molecule as defined in any one of the preceding points, comprising a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO: 29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37 and SEQ ID NO:39 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO: 30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 and SEQ ID NO:40.

(10) The trimeric antigen binding molecule as defined in any one of the preceding points, comprising a moiety capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40 comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:27 and a VL comprising the amino acid sequence of SEQ ID NO:28,

(ii) a VH comprising the amino acid sequence of SEQ ID NO:29 and a VL comprising the amino acid sequence of SEQ ID NO:30,

(iii) a VH comprising the amino acid sequence of SEQ ID NO:31 and a VL comprising the amino acid sequence of SEQ ID NO:32,

(iv) a VH comprising the amino acid sequence of SEQ ID NO:33 and a VL comprising the amino acid sequence of SEQ ID NO:34,

(v) a VH comprising the amino acid sequence of SEQ ID NO:35 and a VL comprising the amino acid sequence of SEQ ID NO:36,

(vi) a VH comprising the amino acid sequence of SEQ ID NO:37 and a VL comprising the amino acid sequence of SEQ ID NO:38, or

(vii) a VH comprising the amino acid sequence of SEQ ID NO:39 and a VL comprising the amino acid sequence of SEQ ID NO:40.

- (11) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:41.

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- (12) The trimeric antigen binding molecule as defined in any one of points (1) to (6), or point (11) above, comprising a moiety capable of specific binding to 4-1BB, wherein the moiety comprises a VH comprising

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(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:42 and SEQ ID NO:43,

(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50,

15 and a VL comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:51 and SEQ ID NO:52,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:53 and SEQ ID NO:54, and

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(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58 and SEQ ID NO:59.

- (13) The trimeric antigen binding molecule as defined in any one of points (1) to (6), point (11) or point (12) above, comprising a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66 and SEQ ID NO:68 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67 and SEQ ID NO:69.

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- (14) The trimeric antigen binding molecule as defined in any one of points (1) to (6), or points (11) to (13) above, comprising a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to 4-1BB comprises
- (i) a VH comprising the amino acid sequence of SEQ ID NO:60 and a VL comprising the amino acid sequence of SEQ ID NO:61,

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- (ii) a VH comprising the amino acid sequence of SEQ ID NO:62 and a VL comprising the amino acid sequence of SEQ ID NO:63,
- (iii) a VH comprising the amino acid sequence of SEQ ID NO:64 and a VL comprising the amino acid sequence of SEQ ID NO:65,
- 5 (iv) a VH comprising the amino acid sequence of SEQ ID NO:66 and a VL comprising the amino acid sequence of SEQ ID NO:67, or
- (v) a VH comprising the amino acid sequence of SEQ ID NO:68 and a VL comprising the amino acid sequence of SEQ ID NO:69.

10 (15) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member is fused at the C-terminal amino acid to the N-terminal amino acid of the trimerization domain, optionally through a peptide linker.

15 (16) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the moiety capable of specific binding to a costimulatory TNF receptor family member is a Fab fragment or a scFv.

20 (17) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the target cell antigen is selected from the group consisting of Fibroblast Activation Protein (FAP), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), Carcinoembryonic Antigen (CEA), CD19, CD20 and CD33.

25 (18) The trimeric antigen binding molecule as defined in any one of the preceding points, comprising a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising

(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:70 and SEQ ID NO:76,

30 (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:71 and SEQ ID NO:77, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:72 and SEQ ID NO:78,

and a VL comprising

35 (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:73 and SEQ ID NO:79,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:74 and SEQ ID NO:80, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75 and SEQ ID NO:81.

(19) The trimeric antigen binding molecule as defined in any one of the preceding points, comprising a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:82 and SEQ ID NO:84, and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:83 and SEQ ID NO:85.

(20) The trimeric antigen binding molecule as defined in any one of the preceding points, comprising a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises
(i) a VH comprising the amino acid sequence of SEQ ID NO:82 and a VL comprising the amino acid sequence of SEQ ID NO:83, or
(ii) a VH comprising the amino acid sequence of SEQ ID NO:84 and a VL comprising the amino acid sequence of SEQ ID NO:85.

(21) The trimeric antigen binding molecule as defined in any one of the preceding points, wherein the moiety capable of specific binding to a target cell antigen is fused at the N-terminal amino acid to the C-terminal amino acid of the trimerization domain, optionally through a peptide linker.

(22) A fusion polypeptide comprising (a) a VH and/or VL of a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) comprising the amino acid sequence of SEQ ID NO:2, wherein the trimerization domain is capable of mediating stable association of the fusion polypeptide with two further such fusion polypeptides and (c) a VH and/or VL of a moiety capable of specific binding to a target cell antigen.

(23) The fusion polypeptide of point (22) above, wherein the fusion polypeptide comprises a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising
(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:70 and SEQ ID NO:76,

(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:71 and SEQ ID NO:77, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:72 and SEQ ID NO:78,

5 and a VL comprising

(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:73 and SEQ ID NO:79,

(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:74 and SEQ ID NO:80, and

10 (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75 and SEQ ID NO:81.

(24) The fusion polypeptide of point (22) or point (23) above, wherein the fusion polypeptide comprises a VH of a moiety capable of specific binding to OX40, wherein the VH comprises

15 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5,

(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and

20 (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14.

(25) The fusion polypeptide of point (22) or point (23) above, wherein the fusion polypeptide comprises a VH of a moiety capable of specific binding to 4-1BB, wherein the VH comprises

25 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:42 and SEQ ID NO:43,

30 (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and

(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50.

(26) A polynucleotide encoding the trimeric antigen binding molecule of any one of points (1) to (21) above, or the fusion polypeptide of any one of points (22) to (25) above.

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(27) An expression vector comprising the polynucleotide of point (26) above.

(28) A host cell comprising the polynucleotide point (26) above or the expression vector of point (27) above.

(29) A method of producing a trimeric antigen binding molecule, comprising culturing the host cell of point (28) above under conditions suitable for the expression of the trimeric antigen binding molecule, and isolating the trimeric antigen binding molecule.

(30) A pharmaceutical composition comprising the trimeric antigen binding molecule of any one of points (1) to (21) above and at least one pharmaceutically acceptable excipient.

(31) The trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above, for use as a medicament.

(32) The trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above, for use

- (i) in stimulating T cell response,
- (ii) in supporting survival of activated T cells,
- (iii) in the treatment of infections,
- (iv) in the treatment of cancer,
- (v) in delaying progression of cancer, or
- (vi) in prolonging the survival of a patient suffering from cancer.

(33) The trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above, for use in the treatment of cancer.

(34) Use of the trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above, in the manufacture of a medicament for the treatment of cancer.

(35) A method of treating an individual having cancer comprising administering to the individual an effective amount of the trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above.

(36) The trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above, for use in up-regulating or prolonging cytotoxic T cell activity.

(37) Use of the trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above, in the manufacture of a medicament for up-regulating or prolonging cytotoxic T cell activity.

5 (38) A method of up-regulating or prolonging cytotoxic T cell activity in an individual having cancer, comprising administering to the individual an effective amount of the trimeric antigen binding molecule of any one of points (1) to (21) above, or the pharmaceutical composition of point (30) above.

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EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Recombinant DNA techniques

Standard methods were used to manipulate DNA as described in Sambrook et al.,
15 Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions. General information regarding the nucleotide sequences of human immunoglobulin light and heavy chains is given in: Kabat, E.A. et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No 91-3242.

20 DNA sequencing

DNA sequences were determined by double strand sequencing.

Gene synthesis

Desired gene segments were either generated by PCR using appropriate templates or were synthesized by Geneart AG (Regensburg, Germany) from synthetic oligonucleotides and PCR
25 products by automated gene synthesis. In cases where no exact gene sequence was available, oligonucleotide primers were designed based on sequences from closest homologues and the genes were isolated by RT-PCR from RNA originating from the appropriate tissue. The gene segments flanked by singular restriction endonuclease cleavage sites were cloned into standard cloning / sequencing vectors. The plasmid DNA was purified from transformed bacteria and
30 concentration determined by UV spectroscopy. The DNA sequence of the subcloned gene fragments was confirmed by DNA sequencing. Gene segments were designed with suitable restriction sites to allow sub-cloning into the respective expression vectors. All constructs were designed with a 5'-end DNA sequence coding for a leader peptide which targets proteins for secretion in eukaryotic cells.

Protein purification

Proteins were purified from filtered cell culture supernatants referring to standard protocols. In brief, antibodies were applied to a Protein A Sepharose column (GE healthcare) and washed with PBS. Elution of antibodies was achieved at pH 2.8 followed by immediate neutralization of the sample. Aggregated protein was separated from monomeric antibodies by size exclusion chromatography (Superdex 200, GE Healthcare) in PBS or in 20 mM Histidine, 150 mM NaCl pH 6.0. Monomeric antibody fractions were pooled, concentrated (if required) using e.g., a MILLIPORE Amicon Ultra (30 MWCO) centrifugal concentrator, frozen and stored at -20°C or -80°C. Part of the samples were provided for subsequent protein analytics and analytical characterization e.g. by SDS-PAGE, size exclusion chromatography (SEC) or mass spectrometry.

SDS-PAGE

The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instruction. In particular, 10% or 4-12% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MES (reduced gels, with NuPAGE® Antioxidant running buffer additive) or MOPS (non-reduced gels) running buffer was used.

Analytical size exclusion chromatography

Size exclusion chromatography (SEC) for the determination of the aggregation and oligomeric state of antibodies was performed by HPLC chromatography. Briefly, Protein A purified antibodies were applied to a Tosoh TSKgel G3000SW column in 300 mM NaCl, 50 mM KH₂PO₄/K₂HPO₄, pH 7.5 on an Agilent HPLC 1100 system or to a Superdex 200 column (GE Healthcare) in 2 x PBS on a Dionex HPLC-System. The eluted protein was quantified by UV absorbance and integration of peak areas. BioRad Gel Filtration Standard 151–1901 served as a standard.

Example 1

Generation of OX40 antibodies

1.1 Preparation, purification and characterization of antigens and screening tools for the generation of novel OX40 binders by Phage Display

DNA sequences encoding the ectodomains of human, mouse or cynomolgus OX40 (Table 1) were subcloned in frame with the human IgG1 heavy chain CH2 and CH3 domains on the knob (Merchant et al., 1998). An AcTEV protease cleavage site was introduced between an antigen ectodomain and the Fc of human IgG1. An Avi tag for directed biotinylation was introduced at the C-terminus of the antigen-Fc knob. Combination of the antigen-Fc knob chain

containing the S354C/T366W mutations, with a Fc hole chain containing the Y349C/T366S/L368A/Y407V mutations allows generation of a heterodimer which includes a single copy of the OX40 ectodomain containing chain, thus creating a monomeric form of Fc-linked antigen (**Figure 1**). **Table 1** shows the amino acid sequences of the various OX40

5 ectodomains. **Table 2** the cDNA and amino acid sequences of monomeric antigen Fc(kih) fusion molecules as depicted in **Figure 1**.

Table 1. Amino acid numbering of antigen ectodomains (ECD) and their origin

SEQ ID NO:	Construct	Origin	ECD
3	human OX40 ECD	Synthesized according to P43489	aa 29-214
129	cynomolgus OX40 ECD	Isolated from cynomolgus blood	aa 29-214
130	murine OX40 ECD	Synthesized according to P47741	aa 10-211

Table 2. cDNA and amino acid sequences of monomeric antigen Fc(kih) fusion molecules
10 (produced by combination of one Fc hole chain with one antigen Fc knob chain)

SEQ ID NO:	Antigen	Sequence
131	Nucleotide sequence Fc hole chain	GACAAACTCACACATGCCACCGTGCCAGCACCT GAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCC CAAAACCCAAGGACACCTCATGATCTCCCGGACCC CTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACG AAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACG GCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGG GAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGC GTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGC AAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTC CCAGCCCCCATCGAGAAAACCATCTCCAAGGCCAAA GGGCAGCCCCGAGAACCACAGGTGTGCACCCTGCC CCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGC CTCTCGTGCGCAGTCAAAGGCTTCTATCCCAGCGAC ATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGA GAACAACACTACAAGACCACGCCTCCCGTGCTGGACTC CGACGGCTCCTTCTTCCTCGTGAGCAAGCTCACCGTG GACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCA TGCTCCGTGATGCATGAGGCTCTGCACAACCACTAC ACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA

132	Nucleotide sequence human OX40 antigen Fc knob chain	CTGCACTGCGTGGGCGACACCTACCCCAGCAACGAC CGGTGCTGCCACGAGTGCAGACCCGGCAACGGCATG GTGTCCCGGTGCAGCCGGTCCCAGAACACCGTGTGC AGACCTTGCGGCCCTGGCTTCTACAACGACGTGGTG TCCAGCAAGCCCTGCAAGCCTTGTACCTGGTGCAAC CTGCGGAGCGGCAGCGAGCGGAAGCAGCTGTGTACC GCCACCCAGGATACCGTGTGCCGGTGTAGAGCCGGC ACCCAGCCCCTGGACAGCTACAAACCCGGCGTGGAC TGCGCCCTTGCCCTCCTGGCCACTTCAGCCCTGGCG ACAACCAGGCCTGCAAGCCTTGGACCAACTGCACCC TGGCCGGCAAGCACACCCTGCAGCCCGCCAGCAATA GCAGCGACGCCATCTGCGAGGACCGGGATCCTCCTG CCACCCAGCCTCAGGAAACCCAGGGCCCTCCCGCCA GACCCATCACCGTGCAGCCTACAGAGGCCTGGCCCA GAACCAGCCAGGGGCCTAGCACCAGACCCGTGGAA GTGCCTGGCGGCAGAGCCGTGACGAACAGTTATAT TTTCAGGGCGGCTCACCCAAATCTGCAGACAAAAC CACACATGCCACCGTGCCAGCACCTGAACTCCTG GGGGGACCGTCAGTCTTCCTCTTCCCCC AAAACCCA AGGACACCCTCATGATCTCCCGGACCCCTGAGGTCA CATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGG TGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAG TACAACAGCACGTACCGTGTGGTCAGCGTCCTCACC GTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCC ATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCC CGAGAACCACAGGTGTACACCCTGCCCCCATGCCGG GATGAGCTGACCAAGAACCAGGTCAGCCTGTGGTGC CTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG GAGTGGGAGAGCAATGGGCAGCCGGAGAACAATA CAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTC CTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAG CAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGT GATGCATGAGGCTCTGCACAACCACTACACGCAGAA GAGCCTCTCCCTGTCTCCGGGTAAATCCGGAGGCCT GAACGACATCTTCGAGGCCCAGAAGATTGAATGGCA CGAG
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133	Nucleotide sequence cynomolgus OX40 antigen Fc knob chain	CTCCACTGTGTGCGGGGACACCTACCCCAGCAACGAC CGGTGCTGTCAGGAGTGCAGGCCAGGCAACGGGATG GTGAGCCGCTGCAACCGCTCCCAGAACACGGTGTGC CGTCCGTGCGGGCCCGGCTTCTACAACGACGTGGTC AGCGCCAAGCCCTGCAAGGCCTGCACATGGTGCAAC CTCAGAAAGTGGGAGTGAGCGGAAACAGCCGTGCAC GGCCACACAGGACACAGTCTGCCGCTGCCGGGCGGG CACCCAGCCCCTGGACAGCTACAAGCCTGGAGTTGA CTGTGCCCCCTGCCCTCCAGGGGCACTTCTCCCCGGGC GACAACCAGGCCTGCAAGCCCTGGACCAACTGCACC TTGGCCGGGAAGCACACCCTGCAGCCAGCCAGCAAT AGCTCGGACGCCATCTGTGAGGACAGGGACCCCCCA CCCACACAGCCCCAGGAGACCCAGGGCCCCCGGCC AGGCCCACCACTGTCCAGCCCCTGAAGCCTGGCCC AGAACCTCACAGAGACCCTCCACCCGGCCCGTGGAG GTCCCCAGGGGCGCCTGCGGTGACGAACAGTTATAT TTTCAGGGCGGCTCACCCAAATCTGCAGACAAAAT CACACATGCCCACCGTGCCAGCACCTGAAGTCTCTG GGGGGACCGTCAGTCTTCTCTTCCCCCCTAAAACCCA AGGACACCCTCATGATCTCCCGGACCCCTGAGGTCA CATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGG TGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAG TACAACAGCACGTACCGTGTGGTCAGCGTCTCTACC GTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCC ATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCC CGAGAACCACAGGTGTACACCCTGCCCCCATGCCGG GATGAGCTGACCAAGAACCAGGTCAGCCTGTGGTGC CTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG GAGTGGGAGAGCAATGGGCAGCCGGAGAACAATA CAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTC CTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAG CAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGT GATGCATGAGGCTCTGCACAACCACTACACGCAGAA GAGCCTCTCCCTGTCTCCGGGTAAATCCGGAGGCCT GAACGACATCTTCGAGGCCAGAAAGATTGAATGGCA CGAG
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134	Nucleotide sequence murine OX40 antigen Fc knob chain	GTGACCGCCAGACGGCTGAACTGCGTGAAGCACACC TACCCCAGCGGCCACAAGTGCTGCAGAGAGTGCCAG CCCGGCCACGGCATGGTGTCCAGATGCGACCACACA CGGGACACCCTGTGCCACCCTTGCGAGACAGGCTTC TACAACGAGGGCCGTGAACTACGATACCTGCAAGCAG TGCACCCAGTGCAACCACAGAAGCGGCAGCGAGCTG AAGCAGAACTGCACCCCCACCCAGGATACCGTGTGC AGATGCAGACCCGGCACCCAGCCAGACAGGACAG CGGCTACAAGCTGGGCGTGGACTGCGTGCCCTGCCC TCCTGGCCACTTCAGCCCCGGCAACAACCAGGCCTG CAAGCCCTGGACCAACTGCACCCTGAGCGGCAAGCA GACCAGACACCCCGCCAGCGACAGCCTGGATGCCGT GTGCGAGGACAGAAGCCTGCTGGCCACCCTGCTGTG GGAGACACAGCGGCCACCTTCAGACCCACCACCGT GCAGAGCACCAACCGTGTGGCCCAGAACCAGCGAGCT GCCCAGTCCTCCTACCCTCGTGACACCTGAGGGCCCC GTCGACGAACAGTTATATTTTCAGGGCGGCTCACCC AAATCTGCAGACAAAACCTCACACATGCCACCCTGC CCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTC CTCTTCCCCCAAAACCAAGGACACCCTCATGATCT CCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACG TGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGT ACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAA AGCCGCGGGAGGAGCAGTACAACAGCACGTACCGT GTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGG CTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGCCCTCCAGCCCCATCGAGAAAACCATCTCC AAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTA CACCCTGCCCCCATGCCGGGATGAGCTGACCAAGAA CCAGGTGAGCCTGTGGTGCCTGGTCAAAGGCTTCTAT CCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG CAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTG CTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGC TCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAAC GTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACA ACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG GTAAATCCGGAGGCCTGAACGACATCTTCGAGGCC AGAAGATTGAATGGCACGAG
135	Fc hole chain	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVK GFYPDSIAVEWESNGQPENNYKTTTPVLDSDGSFFLV KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK

136	human OX40 antigen Fc knob chain	LHCVGDTYPSNDRCCHECRPGNGMVSRCRSQNTVCR PCGPGFYNDVVSSKPCCKPCTWCNLRSGSERKQLCTAT QDTVCRRCRAGTQPLDSYKPGVDCAPCPPGHFSPGDNQ ACKPWTNCTLAGKHTLQPASNSSDAICEDRDPATQPQ ETQGPPARPITVQPTEAWPRTSQQGPSTRPVEVPGGRAV DEQLYFQGGSPKSADKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDE LTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGKSGGLNDIFEAQKIEWHE
137	cynomolgus OX40 antigen Fc knob chain	LHCVGDTYPSNDRCCQECRPGNGMVSRCNRSQNTVCR PCGPGFYNDVVSAPCKACTWCNLRSGSERKQPCTAT QDTVCRRCRAGTQPLDSYKPGVDCAPCPPGHFSPGDNQ ACKPWTNCTLAGKHTLQPASNSSDAICEDRDPPTQPQ ETQGPPARPTTVQPTEAWPRTSQRPPSTRPVEVPRGPAV DEQLYFQGGSPKSADKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDE LTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGKSGGLNDIFEAQKIEWHE
138	murine OX40 antigen Fc knob chain	VTARRLNCVKHTYPSGHKCCRECQPGHGMVSRCDHT RDTLCHPCETGFYNEAVNYDTCKQCTQCNHRSGSELK QNCTPTQDTVCRRCRPGTQPRQDSGYKLGVDVPCPPG HFSPGNNQACKPWTNCTLSGKQTRHPASDSLDAVCE RSLLATLLWETQRPTFRPTTVQSTTVWPRTSELSPPTL VTPEGPVDEQLYFQGGSPKSADKTHTCPPCPAPELLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPE NNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGKSGGLNDIFEAQKIEWHE

All OX40-Fc-fusion encoding sequences were cloned into a plasmid vector driving expression of the insert from an MPSV promoter and containing a synthetic polyA signal sequence located at the 3' end of the CDS. In addition, the vector contained an EBV OriP
5 sequence for episomal maintenance of the plasmid.

For preparation of the biotinylated monomeric antigen/Fc fusion molecules, exponentially growing suspension HEK293 EBNA cells were co-transfected with three vectors encoding the

two components of fusion protein (knob and hole chains) as well as BirA, an enzyme necessary for the biotinylation reaction. The corresponding vectors were used at a 2 : 1 : 0.05 ratio (“antigen ECD-AcTEV- Fc knob” : “Fc hole” : “BirA”).

For protein production in 500 ml shake flasks, 400 million HEK293 EBNA cells were seeded 24 hours before transfection. For transfection, cells were centrifuged for 5 minutes at 210 g, and supernatant was replaced by pre-warmed CD CHO medium. Expression vectors were resuspended in 20 mL of CD CHO medium containing 200 µg of vector DNA. After addition of 540 µL of polyethylenimine (PEI), the solution was vortexed for 15 seconds and incubated for 10 minutes at room temperature. Afterwards, cells were mixed with the DNA/PEI solution, transferred to a 500 mL shake flask and incubated for 3 hours at 37 °C in an incubator with a 5% CO₂ atmosphere. After the incubation, 160 mL of F17 medium was added and cells were cultured for 24 hours. One day after transfection, 1 mM valproic acid and 7% Feed were added to the culture. After 7 days of culturing, the cell supernatant was collected by spinning down cells for 15 min at 210 g. The solution was sterile filtered (0.22 µm filter), supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4 °C.

Secreted proteins were purified from cell culture supernatants by affinity chromatography using Protein A, followed by size exclusion chromatography. For affinity chromatography, the supernatant was loaded on a HiTrap ProteinA HP column (CV = 5 mL, GE Healthcare) equilibrated with 40 mL 20 mM sodium phosphate, 20 mM sodium citrate pH 7.5. Unbound protein was removed by washing with at least 10 column volumes of a buffer containing 20 mM sodium phosphate, 20 mM sodium citrate and 0.5 M sodium chloride (pH 7.5). The bound protein was eluted using a linear pH-gradient of sodium chloride (from 0 to 500 mM) created over 20 column volumes of 20 mM sodium citrate, 0.01% (v/v) Tween-20, pH 3.0. The column was then washed with 10 column volumes of a solution containing 20 mM sodium citrate, 500 mM sodium chloride and 0.01% (v/v) Tween-20, pH 3.0.

The pH of the collected fractions was adjusted by adding 1/40 (v/v) of 2M Tris, pH8.0. The protein was concentrated and filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 2 mM MOPS, 150 mM sodium chloride, 0.02% (w/v) sodium azide solution of pH 7.4.

1.2 Selection of OX40-specific 8H9, 20B7, 49B4, 1G4, CLC-563, CLC-564 and 17A9 antibodies from generic Fab and common light chain libraries

Anti-OX40 antibodies were selected from three different generic phage display libraries: DP88-4 (clones 20B7, 8H9 1G4 and 49B4), the common light chain library Vk3_20/VH3_23 (clones CLC-563 and CLC-564) and lambda-DP47 (clone 17A9).

The **DP88-4 library** was constructed on the basis of human germline genes using the V-domain pairing Vk1_5 (kappa light chain) and VH1_69 (heavy chain) comprising randomized sequence space in CDR3 of the light chain (L3, 3 different lengths) and CDR3 of the heavy chain (H3, 3 different lengths). Library generation was performed by assembly of 3 PCR-amplified fragments applying splicing by overlapping extension (SOE) PCR. Fragment 1 comprises the 5' end of the antibody gene including randomized L3, fragment 2 is a central constant fragment spanning from L3 to H3 whereas fragment 3 comprises randomized H3 and the 3' portion of the antibody gene. The following primer combinations were used to generate these library fragments for DP88-4 library: fragment 1 (forward primer LMB3 combined with reverse primers Vk1_5_L3r_S or Vk1_5_L3r_SY or Vk1_5_L3r_SPY), fragment 2 (forward primer RJH31 combined with reverse primer RJH32) and fragment 3 (forward primers DP88-v4-4 or DP88-v4-6 or DP88-v4-8 combined with reverse primer fdseqlong), respectively. PCR parameters for production of library fragments were 5 min initial denaturation at 94 °C, 25 cycles of 1 min 94 °C, 1 min 58 °C, 1 min 72 °C and terminal elongation for 10 min at 72 °C. For assembly PCR, using equimolar ratios of the gel-purified single fragments as template, parameters were 3 min initial denaturation at 94 °C and 5 cycles of 30 s 94 °C, 1 min 58 °C, 2 min 72 °C. At this stage, outer primers (LMB3 and fdseqlong) were added and additional 20 cycles were performed prior to a terminal elongation for 10 min at 72 °C. After assembly of sufficient amounts of full length randomized Fab constructs, they were digested NcoI / NheI and ligated into similarly treated acceptor phagemid vector. Purified ligations were used for ~60 transformations into electrocompetent E. coli TG1. Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections. These library construction steps were repeated three times to obtain a final library size of 4.4×10^9 . Percentages of functional clones, as determined by C-terminal tag detection in dot blot, were 92.6% for the light chain and 93.7% for the heavy chain, respectively.

The common light chain library Vk3_20/VH3_23 was constructed on the basis of human germline genes using the V-domain pairing Vk3_20 (kappa light chain) and VH3_23 (heavy chain) comprising a constant non-randomized common light chain Vk3_20 and randomized sequence space in CDR3 of the heavy chain (H3, 3 different lengths). Library generation was performed by assembly of 2 PCR-amplified fragments applying splicing by overlapping extension (SOE) PCR. Fragment 1 is a constant fragment spanning from L3 to H3 whereas fragment 2 comprises randomized H3 and the 3' portion of the antibody gene. The following primer combinations were used to generate these library fragments for the Vk3_20/VH3_23 common light chain library: fragment 1 (forward primer MS64 combined with reverse primer DP47CDR3_ba (mod.)) and fragment 2 (forward primers DP47-v4-4, DP47-v4-6, DP47-v4-8 combined with reverse primer fdseqlong), respectively. PCR parameters for production of library fragments were 5 min initial denaturation at 94 °C, 25 cycles of 1 min 94 °C, 1 min 58 °C, 1 min

72 °C and terminal elongation for 10 min at 72 °C. For assembly PCR, using equimolar ratios of the gel-purified single fragments as template, parameters were 3 min initial denaturation at 94 °C and 5 cycles of 30 s 94 °C, 1 min 58 °C, 2 min 72 °C. At this stage, outer primers (MS64 and fdseqlong) were added and additional 18 cycles were performed prior to a terminal elongation for 10 min at 72 °C. After assembly of sufficient amounts of full length randomized VH constructs, they were digested MunI / NotI and ligated into similarly treated acceptor phagemid vector. Purified ligations were used for ~60 transformations into electrocompetent E. coli TG1. Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections. A final library size of 3.75×10^9 was obtained.

Percentages of functional clones, as determined by C-terminal tag detection in dot blot, were 98.9% for the light chain and 89.5% for the heavy chain, respectively.

The **lambda-DP47** library was constructed on the basis of human germline genes using the following V-domain pairings: VL3_19 lambda light chain with VH3_23 heavy chain. The library was randomized in CDR3 of the light chain (L3) and CDR3 of the heavy chain (H3) and was assembled from 3 fragments by “splicing by overlapping extension” (SOE) PCR. Fragment 1 comprises the 5' end of the antibody gene including randomized L3, fragment 2 is a central constant fragment spanning from the end of L3 to the beginning of H3 whereas fragment 3 comprises randomized H3 and the 3' portion of the Fab fragment. The following primer combinations were used to generate library fragments for library: fragment 1 (LMB3 - VL_3_19_L3r_V / VL_3_19_L3r_HV / VL_3_19_L3r_HLV), fragment 2 (RJH80 - DP47CDR3_ba (mod)) and fragment 3 (DP47-v4-4 / DP47-v4-6 / DP47-v4-8 - fdseqlong). PCR parameters for production of library fragments were 5 min initial denaturation at 94 °C, 25 cycles of 60 sec at 94 °C, 60 sec at 55 °C, 60 sec at 72 °C and terminal elongation for 10 min at 72 °C. For assembly PCR, using equimolar ratios of the 3 fragments as template, parameters were 3 min initial denaturation at 94 °C and 5 cycles of 60 sec at 94 °C, 60 sec at 55 °C, 120 sec at 72 °C. At this stage, outer primers were added and additional 20 cycles were performed prior to a terminal elongation for 10 min at 72 °C. After assembly of sufficient amounts of full length randomized Fab fragments, they were digested with NcoI / NheI alongside with similarly treated acceptor phagemid vector. 15ug of Fab library insert were ligated with 13.3ug of phagemid vector. Purified ligations were used for 60 transformations resulting in 1.5×10^9 transformants. Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections.

Human OX40 (CD134) as antigen for the phage display selections was transiently expressed as N-terminal monomeric Fc-fusion in HEK EBNA cells and *in vivo* site-specifically biotinylated via co-expression of BirA biotin ligase at the avi-tag recognition sequence located at the C-terminus of the Fc portion carrying the receptor chain (Fc knob chain).

Selection rounds (biopanning) were performed in solution according to the following pattern:

1. Pre-clearing of ~ 10¹² phagemid particles on maxisorp plates coated with 10 µg/ml of an unrelated human IgG to deplete the libraries of antibodies recognizing the Fc-portion of the antigen,
2. incubation of the non-binding phagemid particles with 100 nM biotinylated human OX40 for 0.5 h in the presence of 100 nM unrelated non-biotinylated Fc knob-into-hole construct for further depletion of Fc-binders in a total volume of 1 ml,
3. capture of biotinylated hu OX40 and attached specifically binding phage by transfer to 4 wells of a neutravidin pre-coated microtiter plate for 10 min (in rounds 1 & 3),
4. washing of respective wells using 5x PBS/Tween20 and 5x PBS,
5. elution of phage particles by addition of 250ul 100 mM TEA (triethylamine) per well for 10 min and neutralization by addition of 500 µl 1M Tris/HCl pH 7.4 to the pooled eluates from 4 wells,
6. post-clearing of neutralized eluates by incubation on neutravidin pre-coated microtiter plate with 100 nM biotin-captured Fc knob-into-hole construct for final removal of Fc-binders,
7. re-infection of log-phase E. coli TG1 cells with the supernatant of eluted phage particles, infection with helperphage VCSM13, incubation on a shaker at 30°C over night and subsequent PEG/NaCl precipitation of phagemid particles to be used in the next selection round.

Selections were carried out over 3 or 4 rounds using constant antigen concentrations of 100 nM. In order to increase the likelihood for binders that are cross-reactive not only to cynomolgus OX40 but also murine OX40, in some selection rounds the murine target was used instead of the human OX40. In rounds 2 and 4, in order to avoid enrichment of binders to neutravidin, capture of antigen : phage complexes was performed by addition of 5.4×10^7 streptavidin-coated magnetic beads. Specific binders were identified by ELISA as follows: 100ul of 25 nM biotinylated human OX40 and 10 µg/ml of human IgG were coated on neutravidin plates and maxisorp plates, respectively. Fab-containing bacterial supernatants were added and binding Fabs were detected via their Flag-tags using an anti-Flag/HRP secondary antibody. Clones exhibiting signals on human OX40 and being negative on human IgG were short-listed for further analyses and were also tested in a similar fashion against cynomolgus and murine OX40. They were bacterially expressed in a 0.5 liter culture volume, affinity purified and further characterized by SPR-analysis using BioRad's ProteOn XPR36 biosensor.

Table 3 shows the sequence of generic phage-displayed antibody library (DP88-4), **Table 4** provides cDNA and amino acid sequences of library DP88-4 germline template and **Table 5** shows the Primer sequences used for generation of DP88-4 germline template.

Table 3. Sequence of generic phage-displayed antibody library (DP88-4)

SEQ ID NO:	Description	Sequence
139	nucleotide sequence of pRJH33 library template DP88-4 library; complete Fab coding region comprising PelB leader sequence + Vk1_5 kappa V-domain + CL constant domain for light chain and PelB + VH1_69 V-domain + CH1 constant domain for heavy chain including tags	TGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTA CTCGCGGCCAGCCGGCCATGGCCGACATCCAGATGACCC AGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACCGTGTG ACCATCACTTGCCGTGCCAGTCAGAGTATTAGTAGCTGGTT GGCCTGGTATCAGCAGAAACCAGGGAAAGCCCCCTAAGCTC CTGATCTATGATGCCTCCAGTTTGGAAAGTGGGGTCCCATC ACGTTTCAGCGGCAGTGGATCCGGGACAGAATTCCTCTC ACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACTTATTA CTGCCAACAGTATAATAGTTATTCTACGTTTGGCCAGGGCA CCAAAGTCGAGATCAAGCGTACGGTGGCTGCACCATCTGT CTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGA GAGGCCAAAGTACAGTGGAAAGGTGGATAACGCCCTCCAAT CGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCA AGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAG CAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAA GTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCT TCAACAGGGGAGAGTGTGGAGCCGCAGAACAAAACTCA TCTCAGAAGAGGATCTGAATGGAGCCGCAGACTACAAGGA CGACGACGACAAGGGTGCCGCATAATAAGGCGCGCCAATT CTATTTCAAGGAGACAGTCATATGAAATACCTGCTGCCGA CCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCAGCCGGCG ATGGCCCAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGA AGAAGCCTGGGTCTCCTCGGTGAAGGTCTCCTGCAAGGCCTC CGGAGGCACATTACAGCAGCTACGCTATAAGCTGGGTGCGA CAGGCCCTGGACAAGGGCTCGAGTGGATGGGAGGGATCA TCCCTATCTTTGGTACAGCAAATACGCACAGAAGTTCCAG GGCAGGGTCACCATTACTGCAGACAAATCCACGAGCACAG CCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACCGC CGTGTATTACTGTGCGAGACTATCCCCAGGCGGTTACTATG TTATGGATGCCTGGGGCCAAGGGACCACCGTGACCGTCTC CTCAGCTAGCACCAAAGGCCCATCGGTCTTCCCCCTGGCAC CCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGG CTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTG TCGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGACACCT TCCCGGCTGTCTACAGTCCTCAGGACTCTACTCCCTCAGC AGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGA CCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAA AGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACGCGGCC GCAAGCACTAGTGCCCATCACCATCACCATCAGCCGCGG CA

Table 4. cDNA and amino acid sequences of library DP88-4 germline template

SEQ ID NO:	Description	Sequence
140	nucleotide sequence of Fab light chain Vk1_5	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTC TGCATCTGTAGGAGACCGTGTCAACATCACTTGCC GTGCCAGTCAGAGTATTAGTAGCTGGTTGGCCTGG TATCAGCAGAAACCAGGGAAAGCCCCCTAAGCTCC TGATCTATGATGCCTCCAGTTTGGAAAGTGGGGTC CCATCACGTTTCAGCGGCAGTGGATCCGGGACAG AATTCACCTCTCACCATCAGCAGCTTGCAGCCTGAT GATTTTGCAACTTATTACTGCCAACAGTATAATAG TTATTCTACGTTTGGCCAGGGCACCAAAGTCGAGA TCAAGCGTACGGTGGCTGCACCATCTGTCTTCATC TTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAC TGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCC CAGAGAGGCCAAAGTACAGTGGAAGGTGGATAAC GCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCA CAGAGCAGGACAGCAAGGACAGCACCTACAGCCT CAGCAGCACCTGACGCTGAGCAAAGCAGACTAC GAGAAACACAAAGTCTACGCCTGCGAAGTCACCC ATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT CAACAGGGGAGAGTGTGGAGCCGCAGAACAAAA ACTCATCTCAGAAGAGGATCTGAATGGAGCCGCA GACTACAAGGACGACGACGACAAGGGTGCCGCA
141	Fab light chain Vk1_5	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQ QKPGKAPKLLIYDASSLESGVPSRFSGSGSGTEFTLT SSLQPDDEFATYYCQQYNSYSTFGQGTKVEIKRTVAA PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW KVDNALQSGNSQESVTEQDSKDSSTLSLTLTKA DYEKHKVYACEVTHQGLSPVTKSFNRGECGAAEQ KLISEEDLNGAADYKDDDDKGAA

142	nucleotide sequence of Fab heavy chain VH1_69	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGA AGAAGCCTGGGTCTCGGTGAAGGTCTCCTGCAA GGCCTCCGGAGGCACATTCAGCAGCTACGCTATA AGCTGGGTGCGACAGGCCCTGGACAAGGGCTCG AGTGGATGGGAGGGATCATCCCTATCTTTGGTACA GCAAACCTACGCACAGAAGTTCCAGGGCAGGGTCA CCATTACTGCAGACAAATCCACGAGCACAGCCTA CATGGAGCTGAGCAGCCTGAGATCTGAGGACACC GCCGTGTATTACTGTGCGAGACTATCCCCAGGCGG TTACTATGTTATGGATGCCTGGGGCCAAGGGACCA CCGTGACCGTCTCCTCAGCTAGCACCAAAGGCCCA TCGGTCTTCCCCCTGGCACCCCTCCTCCAAGAGCAC CTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTC AAGGACTACTTCCCCGAACCGGTGACGGTGTCTGT GGAACCTCAGGCGCCCTGACCAGCGGCGTGCACAC CTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACT CCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGC TTGGGCACCCAGACCTACATCTGCAACGTGAATCA CAAGCCCAGCAACACCAAAGTGGACAAGAAAGTT GAGCCCAAATCTTGTGACGCGGCCGCAAGCACTA GTGCCCATCACCATCACCATCACGCCGCGGCA
143	Fab heavy chain VH1_69	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAISW VRQAPGQGLEWMGGIPIFGTANYAQKFQGRVTITA DKSTSTAYMELSSLRSEDTAVYYCARLSPGGYYVM DAWGQGTITVTVSSASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDK KVEPKSCDAAASTSAHHHHHHAAA

Table 5. Primer sequences used for generation of DP88-4 library

SEQ ID NO:	Primer name	Primer sequence 5' – 3'
144	LMB3	CAGGAAACAGCTATGACCATGATTAC
145	Vk1_5_L3r_S	CTCGACTTTGGTGGCCCTGGCCAAACGTS <i>BAATA</i> <i>CGAATTACT</i> GTTGGCAGTAATAAGTTGCAAAATC AT underlined: 60% original base and 40% randomization as M. bolded and italic: 60% original base and 40% randomization as N

SEQ ID NO:	Primer name	Primer sequence 5' – 3'
146	Vk1_5_L3r_SY	CTCGACTTTGGTGGCCCTGGCCAAACGTM <i>MHRSGRATA</i> <i>CGAATTACT</i> GTTGGCAGTAATAAGTTGCAAAATC AT underlined: 60% original base and 40% randomization as M. bolded and italic: 60% original base and 40% randomization as N
147	Vk1_5_L3r_SPY	CTCGACTTTGGTGGCCCTGGCCAAACGTM <i>MHHMSSSGR</i> <i>ATACGAATTACT</i> GTTGGCAGTAATAAGTTGCAAA ATCAT underlined: 60% original base and 40% randomization as M. bolded and italic: 60% original base and 40% randomization as N
148	RJH31	ACGTTTGGCCAGGGCACCAAAGTCGAG
149	RJH32	TCTCGCACAGTAATACACGGCGGTGTCC
150	DP88-v4-4	GGACACCGCCGTGTATTACTGTGCGAGA-1-2-2-3-4- GAC-TAC- TGGGGCCAAGGGACCACCGTGACCGTCTCC 1: G/D = 20%, E/V/S = 10%, A/P/R/L/T/Y=5%; 2: G/Y/S=15%, A/D/T/R/P/L/V/N/W/F/I/E = 4,6%; 3: G/A/Y = 20%, P/W/S/D/T = 8%; 4: F = 46%, L/M = 15%, G/I/Y = 8%.
151	DP88-v4-6	GGACACCGCCGTGTATTACTGTGCGAGA-1-2-2-2-2-3- 4-GAC-TAC- TGGGGCCAAGGGACCACCGTGACCGTCTCC 1: G/D = 20%, E/V/S = 10%, A/P/R/L/T/Y=5%; 2: G/Y/S=15%, A/D/T/R/P/L/V/N/W/F/I/E = 4,6%; 3: G/A/Y = 20%, P/W/S/D/T = 8%; 4: F = 46%, L/M = 15%, G/I/Y = 8%.
152	DP88-v4-8	GGACACCGCCGTGTATTACTGTGCGAGA-1-2-2-2-2-2- 2-3-4-GAC-TAC- TGGGGCCAAGGGACCACCGTGACCGTCTCC 1: G/D = 20%, E/V/S = 10%, A/P/R/L/T/Y=5%; 2: G/Y/S=15%, A/D/T/R/P/L/V/N/W/F/I/E = 4,6%; 3: G/A/Y =

SEQ ID NO:	Primer name	Primer sequence 5' – 3'
		20%, P/W/S/D/T = 8%; 4: F = 46%, L/M = 15%, G/I/Y = 8%.
153	fdseqlong	GACGTTAGTAAATGAATTTTCTGTATGAGG

Table 6 shows the sequence of generic phage-displayed antibody common light chain library (Vk3_20/VH3_23). **Table 7** provides cDNA and amino acid sequences of common light chain library (Vk3_20/VH3_23) germline template and **Table 8** shows the Primer sequences used for generation of common light chain library (Vk3_20/VH3_23).

Table 6. Sequence of generic phage- displayed antibody common light chain library (Vk3_20/VH3_23) template used for PCR

SEQ ID NO:	Description	Sequence
154	pRJH110 library template of common light chain library Vk3_20/VH3_23 ; complete Fab coding region comprising PelB leader sequence + Vk3_20 kappa V-domain + CL constant domain for light chain and PelB + VH3_23 V-domain + CH1 constant domain for heavy chain including tags	ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATT ACTCGCGGCCCCAGCCGGCCATGGCCGAAATCGTGTTAACG CAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAG CCACCCTCTCTTGCAGGGGCCAGTCAGAGTGTTAGCAGCAG CTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCC AGGCTCCTCATCTATGGAGCATCCAGCAGGGCCACTGGCA TCCCAGACAGGTTTCAGTGGCAGTGGATCCGGGACAGACTT CACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCA GTGTATTACTGTCAGCAGTATGGTAGCTCACCGCTGACGTT CGGCCAGGGGACCAAAGTGGAAATCAAACGTACGGTGGCT GCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTT GAAATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACT TCTATCCCAGAGAGGGCCAAAGTACAGTGGAAAGGTGGATAA CGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAG CAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCC TGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTA CGCCTGCGAAGTCAACCATCAGGGCCTGAGCTCGCCCGTC ACAAAGAGCTTCAACAGGGGAGAGTGTGGAGCCGCACATC ACCATCACCATCACGGAGCCGCAGACTACAAGGACGACGA CGACAAGGGTGCCGCATAATAAGGCGCGCCAATTCTATTT CAAGGAGACAGTCATATGAAATACCTGCTGCCGACCGCTG CTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCC GAGGTGCAATTGCTGGAGTCTGGGGGAGGCTTGGTACAGC CTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTC ACCTTTAGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCC AGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGT GGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGT TCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCT GCAGATGAACAGCCTGAGAGCCGAGGACACGGCCGTATAT TACTGTGCGAAACCGTTTCCGTATTTTGACTACTGGGGCCA AGGAACCCTGGTCACCGTCTCGAGTGCTAGCACCAAAGGC CCATCGGTCTTCCCCCTGGCACCTCCTCCAAGAGCACCTC TGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTAC TTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCC TGACCAGCGGCGTGACACCTTCCCGGCTGTCTTACAGTCC TCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTC CAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAAT CACAAGCCCAGCAACACCAAAGTGGACAAGAAAGTTGAG CCCAAATCTTGTGACGCGGCCGCAGAACAAAACTCATCT CAGAAGAGGATCTGAATGCCGCGGCA

Table 7. cDNA and amino acid sequences of common light chain library (Vk3_20/VH3_23)
germline template

SEQ ID NO:	Description	Sequence
155	nucleotide sequence of Fab light chain Vk3_20	GAAATCGTGTTAACGCAGTCTCCAGGCACCCTGTCTT TGTCTCCAGGGGAAAGAGCCACCCTCTCTTGCAGGG CCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGT ACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCA TCTATGGAGCATCCAGCAGGGCCACTGGCATCCCAG ACAGGTTCAGTGGCAGTGGATCCGGGACAGACTTCA CTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTG CAGTGTATTACTGTCAGCAGTATGGTAGCTCACCGCT GACGTTTCGGCCAGGGGACCAAAGTGGAAATCAAAC GTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCC ATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTT GTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCC AAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCG GGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGC AAGGACAGCACCTACAGCCTCAGCAGCACCTGACG CTGAGCAAAGCAGACTACGAGAAACACAAAGTCTAC GCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCC GTCACAAAGAGCTTCAACAGGGGAGAGTGTGGAGCC GCACATCACCATCACCATCACGGAGCCGCAGACTAC AAGGACGACGACGACAAGGGTGCCGCA
156	Fab light chain Vk3_20	EIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQ KPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRL EPEDFAVYYCQQYGSSPLTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVCLLNFPYFPAKVKQWKVDNAL QSGNSQESVTEQDSKSTYSLSSTLTLSKADYEKHKVY ACEVTHQGLSSPVTKSFNRGECGAAHHHHHHGAADY KDDDDKGAA

157	nucleotide sequence of Fab heavy chain VH3_23	GAGGTGCAATTGCTGGAGTCTGGGGGAGGCTTGGTA CAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCC TCCGGATTACCTTTAGCAGTTATGCCATGAGCTGGG TCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCT CAGCTATTAGTGGTAGTGGTGGTAGCACATACTACG CAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAG ACAATTCCAAGAACACGCTGTATCTGCAGATGAACA GCCTGAGAGCCGAGGACACGGCCGTATATTACTGTG CGAAACCGTTTCCGTATTTTGACTACTGGGGCCAAG GAACCCTGGTCACCGTCTCGAGTGCTAGCACCAAAG GCCCATCGGTCTTCCCCCTGGCACCCCTCCTCCAAGAG CACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGT CAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTG GAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTT CCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTC AGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGC ACCCAGACCTACATCTGCAACGTGAATCACAAGCCC AGCAACACCAAAGTGGACAAGAAAGTTGAGCCCAA ATCTTGTGACGCGGCCCGCAGAACAAAACTCATCTC AGAAGAGGATCTGAATGCCGCGGCA
158	Fab heavy chain VH3_23 (DP47)	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWV RQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAKPFYFDYWQGGL VTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDAAAEQ KLISEEDLNAAA

Table 8. Primer sequences used for generation of common light chain library (Vk3_20/VH3_23)

SEQ ID NO:	Primer name	Primer sequence 5' – 3'
159	MS64	ACGTTCGGCCAGGGGACCAAAGTGG
160	DP47CDR3_ba (mod.)	CGCACAGTAATATACGGCCGTGTCC
161	DP47-v4-4	CGAGGACACGGCCGTATATTACTGTGCG-5-1-2-2-3-4- GAC-TAC-TGGGGCCAAGGAACCCTGGTCACCGTCTCG
162	DP47-v4-6	CGAGGACACGGCCGTATATTACTGTGCG-5-1-2-2-2-3- 4-GAC-TAC- TGGGGCCAAGGAACCCTGGTCACCGTCTCG

SEQ ID NO:	Primer name	Primer sequence 5' – 3'
163	DP47-v4-8	CGAGGACACGGCCGTATATTACTGTGCG-5-1-2-2-2-2-2-2-3-4-GAC-TAC-TGGGGCCAAGGAACCCTGGTCACCGTCTCG
164	fdseqlong	GACGTTAGTAAATGAATTTTCTGTATGAGG

1: G/D = 20%, E/V/S = 10%, A/P/R/L/T/Y=5%; 2: G/Y/S=15%, A/D/T/R/P/L/V/N/W/F/I/E = 4,6%; 3: G/A/Y = 20%, P/W/S/D/T = 8%; 4: F = 46%, L/M = 15%, G/I/Y = 8%; 5: K=70%, R =30%.

Table 9 shows the sequence of generic phage-displayed lambda-DP47 library

5 (V13_19/VH3_23) template used for PCRs. **Table 10** provides cDNA and amino acid sequences of lambda-DP47 library (V13_19/VH3_23) germline template and **Table 11** shows the Primer sequences used for generation of lambda-DP47 library (V13_19/VH3_23).

Table 9: Sequence of generic phage- displayed lambda-DP47 library (V13_19/VH3_23) template used for PCRs

SEQ ID NO:	Description	Sequence
165	pRJH53 library template of lambda-DP47 library V13_19/VH3_23 ; complete Fab coding region comprising PelB leader sequence + V13_19 lambda V-domain + CL constant domain for light chain and PelB + VH3_23 V-domain + CH1 constant domain for heavy chain including tags	ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATT ACTCGCGGCCCCAGCCGGCCATGGCCTCGTCTGAGCTGACT CAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCA GGATCACATGCCAAGGAGACAGCCTCAGAAGTTATTATGC AAGCTGGTACCAGCAGAAGCCAGGACAGGCCCTGTACTT GTCATCTATGGTAAAAACAACCGGCCCTCAGGGATCCAG ACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTTG ACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGACTATT ACTGTAACCTCCCGTGATAGTAGCGGTAATCATGTGGTATTC GGCGGAGGGACCAAGCTGACCGTCCTAGGACAACCCAAG GCTGCCCCCAGCGTGACCCTGTTCCCCCCCAGCAGCGAGG AATTGCAGGCCAACAAAGGCCACCCTGGTCTGCCTGATCAG CGACTTCTACCCAGGCGCCGTGACCGTGGCCTGGAAGGCC GACAGCAGCCCCGTGAAGGCCGGCGTGAGAGACCACCACC CCAGCAAGCAGAGCAACAACAAGTACGCCGCCAGCAGCT ACCTGAGCCTGACCCCCGAGCAGTGGAAGAGCCACAGGTC CTACAGCTGCCAGGTGACCCACGAGGGCAGCACCGTGGAG AAAACCGTGGCCCCCACCAGGTGCAGCGGAGCCGCAGAAC AAAAACTCATCTCAGAAGAGGATCTGAATGGAGCCGCAGA CTACAAGGACGACGACGACAAGGGTGCCGCATAATAAGG CGCGCCAATTCTATTTCAAGGAGACAGTCATATGAAATAC CTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGC CCAGCCGGCGATGGCCGAGGTGCAATTGCTGGAGTCTGGG GGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCT GTGCAGCCTCCGGATTACCTTTAGCAGTTATGCCATGAGC TGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCT CAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGA CTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCC AAGAACACGCTGTATCTGCAGATGAACAGCCTGAGAGCCG AGGACACGGCCGTATATTACTGTGCGAAACCGTTTCCGTAT TTTGACTACTGGGGCCAAGGAACCCTGGTCACCGTCTCGA GTGCTAGCACCAAGGCCCATCGGTCTTCCCCCTGGCACCC TCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCT GCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTC GTGGAACCTCAGGCGCCCTGACCAGCGGCGTGACACCTTC CCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAG CGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACC TACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAAG TGGACAAGAAAGTTGAGCCCAAATCTTGTGACGCGGCCGC AAGCACTAGTGCCCATCACCATCACCATCACGCCGCGGCA

Table 10. cDNA and amino acid sequences of lambda-DP47 library (V13_19/VH3_23) germline template

SEQ ID NO:	Description	Sequence
166	nucleotide sequence of Fab light chain V13_19	TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGG CCTTGGGACAGACAGTCAGGATCACATGCCAAGGAG ACAGCCTCAGAAGTTATTATGCAAGCTGGTACCAGC AGAAGCCAGGACAGGCCCTGTACTTGTTCATCTATG GTAAAAACAACCGGCCCTCAGGGATCCCAGACCGAT TCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGAC CATCACTGGGGCTCAGGCGGAAGATGAGGCTGACTA TTACTGTAACCTCCCGTGATAGTAGCGGTAATCATGTG GTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGA CAACCCAAGGCTGCCCCCAGCGTGACCCTGTTCCCC CCCAGCAGCGAGGAATTGCAGGCCAACAAAGGCCACC CTGGTCTGCCTGATCAGCGACTTCTACCCAGGCGCCG TGACCGTGGCCTGGAAGGCCGACAGCAGCCCCGTGA AGGCCGGCGTGGAGACCACCACCCCCAGCAAGCAG AGCAACAACAAGTACGCCGCCAGCAGCTACCTGAGC CTGACCCCCGAGCAGTGGAAGAGCCACAGGTCCTAC AGCTGCCAGGTGACCCACGAGGGGCAGCACCGTGGAG AAAACCGTGGCCCCCACCAGGTGCAGCGGAGCCGCA GAACAAAAACTCATCTCAGAAGAGGATCTGAATGGA GCCGCAGACTACAAGGACGACGACGACAAGGGTGC CGCA
167	Fab light chain V13_19	SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQ KPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITG AQAEDEADYYCNSRDSSGNHVVFGGGKLTVLGQPK AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAW KADSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQW KSHRSYSCQVTHEGSTVEKTVAPTECSGA AEQKLISEE DLNGAADYKDDDDKGAA
150	nucleotide sequence of Fab heavy chain VH3_23	see Table 7
151	Fab heavy chain VH3_23 (DP47)	see Table 7

Table 11. Primer sequences used for generation of lambda-DP47 library (Vl3_19/VH3_23)

SEQ ID NO:	Primer name	Primer sequence 5' – 3'
168	LMB3	CAGGAAACAGCTATGACCATGATTAC
169	Vl_3_19_L3r_V	GGACGGTCAGCTTGGTCCCTCCGCCGAATAC <u>VHV</u> <i>ATT</i> <i>ACC</i> <u>GCT</u> <i>ACT</i> <i>ATC</i> <i>ACG</i> GGAGTTACAGTAATAGTCAGCCTCATCTTCCGC underlined: 60% original base and 40% randomization as M bold and italic: 60% original base and 40% randomization as N
170	Vl_3_19_L3r_HV	GGACGGTCAGCTTGGTCCCTCCGCCGAATAC <u>CMM</u> <i>ATG</i> <i>ATT</i> <i>ACC</i> <u>GCT</u> <i>ACT</i> <i>ATC</i> <i>ACG</i> GGAGTTACAGTAATAGTCAGCCTCATCTTCCGC underlined: 60% original base and 40% randomization as M bolded and italic: 60% original base and 40% randomization as N
171	Vl_3_19_L3r_HL V	GGACGGTCAGCTTGGTCCCTCCGCCGAATAC <u>RHM</u> <i>VWG</i> <i>ATG</i> <i>ATT</i> <i>ACC</i> <u>GCT</u> <i>ACT</i> <i>ATC</i> <i>ACG</i> GGAGTTACAGTAATAGTCAGCCTCATCTTCCGC underlined: 60% original base and 40% randomization as M bolded and italic: 60% original base and 40% randomization as N
172	RJH80	TTCGGCGGAGGGACCAAGCTGACCGTCC
195	MS63	TTTCGCACAGTAATATACGGCCGTGTCC

Additional primers used for construction of the lambda-DP47 library, i.e. DP47CDR3_ba (mod.), DP47-v4-4, DP47-v4-6, DP47-v4-8 and fdseqlong, are identical to the primers used for the construction of the common light chain library (Vk3_20/VH3_23) and have already been listed in **Table 8**.

Clones 8H9, 20B7, 49B4, 1G4, CLC-563, CLC-564 and 17A9 were identified as human OX40-specific binders through the procedure described above. The cDNA sequences of their variable regions are shown in **Table 12** below, the corresponding amino acid sequences can be found in **Table C**.

Table 12. Variable region base pair sequences for phage-derived anti-OX40 antibodies. Underlined are the complementarity determining regions (CDRs).

Clone	SEQ ID NO:	Sequence
8H9	173 (VL)	TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGG GACAGACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA <u>AGTTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCC</u> CCTGTACTTGTCTATCTATGGTAAAAACAACCGGCCCTCAGGG ATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCT TCCTTGACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGAC TATTACTGTAACTCCCGTGTTATGCCTCATAATCGCGTATTCTG GCGGAGGGACCAAGCTGACCGTC
	174 (VH)	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCT GGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTACCT TTAGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGA AGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTA <u>GCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCT</u> CCAGAGACAATTCCAAGAACACGCTGTATCTGCAGATGAACA GCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGCGTG <u>TTTTCTACCGTGGTGGTGGTCTTCTATGGACTACTGGGGCCAAGG</u> AACCTGGTCAACCGTCTCGAGT
49B4	175 (VL)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTG TAGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGAAAGTGG GGTCCCATCACGTTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGTATAGTTTCGCAGCCGTATACGTTTGGCC AGGGCACCAAAGTCGAGATCAAG
	176 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACACTACGCACAGAAGTTCCAGGGCAGGGTCACCAT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>GAATACTACCGTGGTCCGTACGACTACTGGGGCCAAGGGACC</u> ACCGTGACCGTCTCCTCA

1G4	177 (VL)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTG TAGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGG GGTCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCAACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGTATATTTTCGTATTCCATGTTGACGTTTG GCCAGGGCACCAAAGTCGAGATCAAG
	178 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACACTACGCACAGAAAGTTCCAGGGCAGGGTCACCATT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>GAATACGGTTCTATGGACTACTGGGGCCAAGGGACCACCGTG</u> ACCGTCTCCTCA
20B7	179 (VL)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTG TAGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGG GGTCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCAACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGTATCAGGCTTTTTTCGCTTACGTTTGGCC AGGGCACCAAAGTCGAGATCAAG
	180 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACACTACGCACAGAAAGTTCCAGGGCAGGGTCACCATT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>GTAACTACCCGTACTCTTACTGGGGTGACTTCGACTACTGGG</u> GCCAAGGGACCACCGTGACCGTCTCCTCA
CLC-563	181 (VL)	GAGATCGTGCTGACCCAGAGCCCCGGCACACTCTCCCTGTCTC CTGGGGAAAGGGCCACCCTTTCATGCAGAGCCAGCCAGTCCG <u>TCTCTAGTAGCTACCTGGCATGGTATCAGCAGAAAGCCAGGAC</u> AAGCCCCCGCCTCCTGATTTACGGCGCTTCCTCTCGGGCAAC <u>TGGTATCCCTGACAGGTTCTCAGGGAGCGGAAGCGGAACAGA</u> TTTTACCTTGACTATTTCTAGACTGGAGCCAGAGGACTTCGCC GTGTATTACTGT <u>CAGCAGTACGGTAGTAGCCCCCTCACCTTTG</u> GCCAGGGGACAAAAGTCGAAATCAAG

	182 (VH)	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCT GGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTACCT TTAGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGA AGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTA <u>GCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCT</u> CCAGAGACAATTCCAAGAACACGCTGTATCTGCAGATGAACA GCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGCTTG <u>ACGTTGGTGCTTTCGACTACTGGGGCCAAGGAGCCCTGGTCA</u> CCGTCTCGAGT
CLC- 564	183 (VL)	GAGATCGTGCTGACCCAGAGCCCCGGCACACTCTCCCTGTCTC CTGGGGAAAGGGGCCACCCTTTCATGCAGAGCCAGCCAGTCCG <u>TCTCTAGTAGCTACCTGGCATGGTATCAGCAGAAGCCAGGAC</u> AAGCCCCCGCCTCCTGATTTACGGCGCTTCCTCTCGGGCAAC <u>TGGTATCCCTGACAGGTTCTCAGGGAGCGGAAGCGGAACAGA</u> TTTTACCTTGACTATTTCTAGACTGGAGCCAGAGGACTTCGCC GTGTATTACTGT <u>CAGCAGTACGGTAGTAGCCCCCTCACCTTTG</u> GCCAGGGGACAAAAGTCGAAATCAAG
	184 (VH)	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCT GGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTACCT TTAGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGA AGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTA <u>GCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCT</u> CCAGAGACAATTCCAAGAACACGCTGTATCTGCAGATGAACA GCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGTTG <u>ACGTTGGTCCGTTCGACTACTGGGGCCAAGGAACCCTGGTCA</u> CCGTCTCGAGT
17A9	185 (VL)	TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGG GACAGACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA <u>AGTTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCC</u> CCTGTACTTGTCTATCTATGGTAAAAACAACCGGCCCTCAGGG ATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCT TCCTTGACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGAC TATTACTGTAACTCCCGTGTTATGCCTCATAATCGCGTATTG GCGGAGGGACCAAGCTGACCGTC
	186 (VH)	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCT GGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTACCT TTAGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGA AGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTA <u>GCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCT</u> CCAGAGACAATTCCAAGAACACGCTGTATCTGCAGATGAACA GCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGCGTG <u>TTTTCTACCGTGGTGGTGGTTTCTATGGACTACTGGGGCCAAGG</u> AACCTGGTCAACCGTCTCGAGT

Example 2

Generation of 4-1BB antibodies

2.1 Preparation, purification and characterization of antigens and screening tools for the generation of novel 4-1BB binders by Phage Display

DNA sequences encoding the ectodomains of human, mouse or cynomolgus 4-1BB (**Table 13**) were subcloned in frame with the human IgG1 heavy chain CH2 and CH3 domains on the knob (Merchant et al., 1998). An AcTEV protease cleavage site was introduced between an antigen ectodomain and the Fc of human IgG1. An Avi tag for directed biotinylation was introduced at the C-terminus of the antigen-Fc knob. Combination of the antigen-Fc knob chain containing the S354C/T366W mutations, with a Fc hole chain containing the Y349C/T366S/L368A/Y407V mutations allows generation of a heterodimer which includes a single copy of 4-1BB ectodomain containing chain, thus creating a monomeric form of Fc-linked antigen (**Figure 1**). **Table 14** shows the cDNA and amino acid sequences of the antigen Fc-fusion constructs.

Table 13. Amino acid numbering of antigen ectodomains (ECD) and their origin

SEQ ID NO:	Construct	Origin	ECD
41	human 4-1BB ECD	Synthetized according to Q07011	aa 24-186
187	cynomolgus 4-1BB ECD	isolated from cynomolgus blood	aa 24-186
188	murine 4-1BB ECD	Synthetized according to P20334	aa 24-187

Table 14. cDNA and amino acid sequences of monomeric antigen Fc(kih) fusion molecules (produced by combination of one Fc hole chain with one antigen Fc knob chain)

SEQ ID NO:	Antigen	Sequence
131	Nucleotide sequence Fc hole chain	see Table 2

189	Nucleotide sequence human 4-1BB antigen Fc knob chain	CTGCAGGACCCCTGCAGCAACTGCCCTGCCGGCACC TTCTGCGACAACAACCGGAACCAGATCTGCAGCCCC TGCCCCCCCCAACAGCTTCAGCTCTGCCGGCGGACAG CGGACCTGCGACATCTGCAGACAGTGCAAGGGCGTG TTCAGAACCCGGAAAGAGTGCAGCAGCACCAGCAAC GCCGAGTGCGACTGCACCCCCGGCTTCCATTGTCTGG GAGCCGGCTGCAGCATGTGCGAGCAGGACTGCAAGC AGGGCCAGGAACTGACCAAGAAGGGCTGCAAGGAC TGCTGCTTCGGCACCTTCAACGACCAGAAGCGGGGC ATCTGCCGGCCCTGGACCAACTGTAGCCTGGACGGC AAGAGCGTGCTGGTCAACGGCACCAAAGAACGGGA CGTCGTGTGCGGCCCCAGCCCTGCTGATCTGTCTCCT GGGGCCAGCAGCGTGACCCCTCCTGCCCTGCCAGA GAGCCTGGCCACTCTCCTCAGGTCGACGAACAGTTA TATTTTCAGGGCGGCTCACCCAAATCTGCAGACAAA ACTCACACATGCCCACCGTGCCAGCACCTGAAGTC CTGGGGGGGACCGTCAGTCTTCCTCTTCCCCCCTGAGG CCAAGGACACCCTCATGATCTCCCGGACCCCTGAGG TCACATGCGTGGTGGTGGACGTGAGCCACGAAGACC CTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGG AGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAG CAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTC ACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAG TACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCC CCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAG CCCCGAGAACCACAGGTGTACACCCTGCCCCCATGC CGGGATGAGCTGACCAAGAACCAGGTCAGCCTGTGG TGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCC GTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAA CTACAAGACCACGCCTCCCGTGCTGGACTCCGACGG CTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAG AGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCC GTGATGCATGAGGCTCTGCACAACCACTACACGCAG AAGAGCCTCTCCCTGTCTCCGGGTAAATCCGGAGGC CTGAACGACATCTTCGAGGCCCAGAAGATTGAATGG CACGAG
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190	Nucleotide sequence cynomolgus 4- 1BB antigen Fc knob chain	TTGCAGGATCTGTGTAGTAACTGCCCAGCTGGTACAT TCTGTGATAATAACAGGAGTCAGATTTGCAGTCCCT GTCCTCCAAATAGTTTCTCCAGCGCAGGTGGACAAA GGACCTGTGACATATGCAGGCAGTGTAAGGTGTTT TCAAGACCAGGAAGGAGTGTTCCCTCCACCAGCAATG CAGAGTGTGACTGCATTTCAGGGTATCACTGCCTGG GGGCAGAGTGCAGCATGTGTGAACAGGATTGTAAAC AAGGTCAAGAATTGACAAAAAAGGTTGTAAAGACT GTTGCTTTGGGACATTTAATGACCAGAAACGTGGCA TCTGTGCCCCCTGGACAAACTGTTCTTTGGATGGAAA GTCTGTGCTTGTGAATGGGACGAAGGAGAGGGACGT GGTCTGCGGACCATCTCCAGCCGACCTCTCTCCAGG AGCATCCTCTGCGACCCCGCCTGCCCCCTGCGAGAGA GCCAGGACACTCTCCGCAGGTGCGACGAACAGTTATA TTTTCAGGGGCGGCTCACCCAAATCTGCAGACAAAAC TCACACATGCCCACCGTGCCCAGCACCTGAACTCCT GGGGGGACCGTCAGTCTTCCTCTTCCCCCCTGAGGTC AAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCT GAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAG GTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCA GTACAACAGCACGTACCGTGTGGTCAGCGTCCTCAC CGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCC CATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCC CCGAGAACCACAGGTGTACACCCTGCCCCCATGCCG GGATGAGCTGACCAAGAACCAGGTCAGCCTGTGGTG CCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGT GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCT ACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCT CCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGA GCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCG TGATGCATGAGGCTCTGCACAACCACTACACGCAGA AGAGCCTCTCCCTGTCTCCGGGTAAATCCGGAGGCC TGAACGACATCTTCGAGGCCCAGAAGATTGAATGGC ACGAG
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191	Nucleotide sequence murine 4-1BB antigen Fc knob chain	GTGCAGAACAGCTGCGACAACCTGCCAGCCCCGGCACC TTCTGCCGGAAGTACAACCCCGTGTGCAAGAGCTGC CCCCCAGCACCTTCAGCAGCATCGGCGGCCAGCCC AACTGCAACATCTGCAGAGTGTGCGCCGGCTACTTC CGGTTCAAGAAGTTCTGCAGCAGCACCCACAACGCC GAGTGCGAGTGCATCGAGGGCTTCCACTGCCTGGGC CCCCAGTGCACCAGATGCGAGAAGGACTGCAGACCC GGCCAGGAAGTGAACCAAGCAGGGGCTGTAAGACCTGC AGCCTGGGCACCTTCAACGACCAGAACGGGACCGGC GTGTGCCGGCCTTGGACCAATTGCAGCCTGGACGGG AGAAGCGTGCTGAAAACCGGCACCACCGAGAAGGA CGTCGTGTGCGGCCCTCCCGTGGTGTCTTCAGCCCT AGCACCACCATCAGCGTGACCCCTGAAGGCGGCCCT GGCGGACACTCTCTGCAGGTCTGGTCGACGAACAG TTATATTTTCAGGGCGGCTCACCCAAATCTGCAGACA AAATCACACATGCCCACCGTGCCCAGCACCTGAAC TCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCAA ACCCAAGGACACCCTCATGATCTCCCGGACCCCTGA GGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGA CCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGT GGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGG AGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCC TCACCGTCTGCACCAGGACTGGCTGAATGGCAAGG AGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAG CCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGC AGCCCCGAGAACCACAGGTGTACACCCTGCCCCCAT GCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGT GGTGCCTGGTCAAAGGCTTCTATCCAGCGACATCG CCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAAC AACTACAAGACCACGCCTCCCGTGCTGGACTCCGAC GGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACA AGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCT CCGTGATGCATGAGGCTCTGCACAACCACTACACGC AGAAGAGCCTCTCCCTGTCTCCGGGTAAATCCGGAG GCCTGAACGACATCTTCGAGGGCCAGAAGATTGAAT GGCACGAG
135	Fc hole chain	see Table 2
192	human 4-1BB antigen Fc knob chain	LQDPCSNCPAGTFCDNNRNQICSPCPPNSFSSAGGQRTC DICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGCS MCEQDCKQGQELTKKGCKDCCFGTFNDQKRIGICRPW TNCSLDGKSVLVNGTKERDVVCGPSPADLSPGASSVTP PAPAREPGHSPQVDEQLYFQGGSPKSADKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP REPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVE WESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKSGGLNDIF EAQKIEWHE

193	cynomolgus 4-1BB antigen Fc knob chain	LQDLCSNCPAGTFCDNRSQICSPCPPNSFSSAGGQRTC DICRQCKGVFKTRKECSSTSNAECDICISGYHCLGAEC MCEQDCKQGQELTKKGCKDCCFGTFNDQKRIGICRPW TNCSLDGKSVLVNGTKERDVVCGPSPADLSPGASSATP PAPAREPGHSPQVDEQLYFQGGSPKSADKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP REPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVE WESNGQPENNYKTTPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKSGGLNDIF EAQKIEWHE
914	murine 4-1BB antigen Fc knob chain	VQNSCDNCQPGTFCRKYNPVCKSCPPSTFSSIGGQPNC NICRVCAGYFRKKFCSSTHNAECECIEGFHCLGPQCTR CEKDCRPGQELTKQGCKTCSLGTENDQNGTGVCRPWT NCSLDGRSVLKTGTTEKD VVCGPPVVSFSPSTTISVTPE GGPGGHS LQVLVDEQLYFQGGSPKSADKTHTCPPCPAP ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWE SNGQPENNYKTTPVLDSDGSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPGKSGGLNDIFE AQKIEWHE

All 4-1BB-Fc-fusion molecule encoding sequences were cloned into a plasmid vector, which drives expression of the insert from an MPSV promoter and contains a synthetic polyA signal sequence located at the 3' end of the CDS. In addition, the vector contains an EBV OriP
5 sequence for episomal maintenance of the plasmid.

For preparation of the biotinylated monomeric antigen/Fc fusion molecules, exponentially growing suspension HEK293 EBNA cells were co-transfected with three vectors encoding the two components of fusion protein (knob and hole chains) as well as BirA, an enzyme necessary for the biotinylation reaction. The corresponding vectors were used at a 2 : 1 : 0.05 ratio
10 ("antigen ECD-AcTEV- Fc knob" : "Fc hole" : "BirA").

For protein production in 500 ml shake flasks, 400 million HEK293 EBNA cells were seeded 24 hours before transfection. For transfection cells were centrifuged for 5 minutes at 210 g, and the supernatant was replaced by pre-warmed CD CHO medium. Expression vectors were resuspended in 20 mL of CD CHO medium containing 200 µg of vector DNA. After addition of
15 540 µL of polyethylenimine (PEI), the solution was vortexed for 15 seconds and incubated for 10 minutes at room temperature. Afterwards, cells were mixed with the DNA/PEI solution, transferred to a 500 mL shake flask and incubated for 3 hours at 37°C in an incubator with a 5 % CO₂ atmosphere. After the incubation, 160 mL of F17 medium was added and cells were

cultured for 24 hours. The production medium was supplemented with 5 μ M kifunensine. One day after transfection, 1 mM valproic acid and 7 % Feed were added to the culture. After 7 days of culturing, the cell supernatant was collected by spinning down cells for 15 min at 210 g. The solution was sterile filtered (0.22 μ m filter), supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4°C.

Secreted proteins were purified from cell culture supernatants by affinity chromatography using Protein A, followed by size exclusion chromatography. For affinity chromatography, the supernatant was loaded on a HiTrap ProteinA HP column (CV = 5 mL, GE Healthcare) equilibrated with 40 mL 20 mM sodium phosphate, 20 mM sodium citrate pH 7.5. Unbound protein was removed by washing with at least 10 column volumes of 20 mM sodium phosphate, 20 mM sodium citrate, 0.5 M sodium chloride containing buffer (pH 7.5). The bound protein was eluted using a linear pH-gradient of sodium chloride (from 0 to 500 mM) created over 20 column volumes of 20 mM sodium citrate, 0.01 % (v/v) Tween-20, pH 3.0. The column was then washed with 10 column volumes of 20 mM sodium citrate, 500 mM sodium chloride, 0.01 % (v/v) Tween-20, pH 3.0. The pH of collected fractions was adjusted by adding 1/40 (v/v) of 2M Tris, pH8.0. The protein was concentrated and filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 2mM MOPS, 150 mM sodium chloride, 0.02 % (w/v) sodium azide solution of pH 7.4.

2.2 Selection of 4-1BB-specific 12B3, 25G7, 11D5, 9B11 and 20G2 antibodies from generic F(ab) libraries

The antibodies **11D5**, **9B11**, and **12B3** with specificity for human and cynomolgus 4-1BB were selected from a generic phage-displayed antibody library (DP88-4) in the Fab format. From the same library, an additional antibody, clone **20G2**, with reactivity to murine 4-1BB was selected as well. This library was constructed on the basis of human germline genes using the V-domain pairing Vk1_5 (kappa light chain) and VH1_69 (heavy chain) comprising randomized sequence space in CDR3 of the light chain (L3, 3 different lengths) and CDR3 of the heavy chain (H3, 3 different lengths). Library generation was performed by assembly of 3 PCR-amplified fragments applying splicing by overlapping extension (SOE) PCR. Fragment 1 comprises the 5' end of the antibody gene including randomized L3, fragment 2 is a central constant fragment spanning from L3 to H3 whereas fragment 3 comprises randomized H3 and the 3' portion of the antibody gene. The following primer combinations were used to generate these library fragments for DP88-4 library: fragment 1 (forward primer LMB3 combined with reverse primers Vk1_5_L3r_S or Vk1_5_L3r_SY or Vk1_5_L3r_SPY), fragment 2 (forward primer RJH31 combined with reverse primer RJH32) and fragment 3 (forward primers DP88-v4-4 or DP88-v4-6 or DP88-v4-8 combined with reverse primer fdseqlong), respectively. PCR parameters for production of library fragments were 5 min initial denaturation at 94 °C, 25

cycles of 1 min 94 °C, 1 min 58 °C, 1 min 72 °C and terminal elongation for 10 min at 72 °C. For assembly PCR, using equimolar ratios of the gel-purified single fragments as template, parameters were 3 min initial denaturation at 94 °C and 5 cycles of 30 s 94 °C, 1 min 58 °C, 2 min 72 °C. At this stage, outer primers (LMB3 and fdseqlong) were added and additional 20
5 cycles were performed prior to a terminal elongation for 10 min at 72 °C. After assembly of sufficient amounts of full length randomized Fab constructs, they were digested *NcoI* / *NheI* and ligated into similarly treated acceptor phagemid vector. Purified ligations were used for ~60 transformations into electrocompetent *E. coli* TG1. Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections. These library
10 construction steps were repeated three times to obtain a final library size of 4.4×10^9 . Percentages of functional clones, as determined by C-terminal tag detection in dot blot, were 92.6% for the light chain and 93.7% for the heavy chain, respectively.

The antibody **25G7** with specificity for human and cynomolgus 4-1BB was selected from a generic phage-displayed antibody library (λ -DP47) in the Fab format. This library was
15 constructed on the basis of human germline genes using the V-domain pairing VL3_19 (lambda light chain) and VH3_23 (heavy chain) comprising randomized sequence space in CDR3 of the light chain (L3, 3 different lengths) and CDR3 of the heavy chain (H3, 3 different lengths). Library generation was performed by assembly of 3 PCR-amplified fragments applying splicing by overlapping extension (SOE) PCR. Fragment 1 comprises the 5' end of the antibody gene
20 including randomized L3, fragment 2 is a central constant fragment spanning from L3 to H3 whereas fragment 3 comprises randomized H3 and the 3' portion of the antibody gene. The following primer combinations were used to generate these library fragments for λ -DP47 library: fragment 1 (forward primer LMB3 combined with reverse primers VL_3_19_L3r_V or VL_3_19_L3r_HV or VL_3_19_L3r_HLV), fragment 2 (forward primer RJH80 combined with
25 reverse primer MS63) and fragment 3 (forward primers DP47-v4-4 or DP47-v4-6 or DP47-v4-8 combined with reverse primer fdseqlong), respectively. PCR parameters for production of library fragments were 5 min initial denaturation at 94 °C, 25 cycles of 1 min 94 °C, 1 min 58 °C, 1 min 72 °C and terminal elongation for 10 min at 72 °C. For assembly PCR, using equimolar ratios of the gel-purified single fragments as template, parameters were 3 min initial denaturation at 94 °C
30 and 5 cycles of 30 s 94 °C, 1 min 58 °C, 2 min 72 °C. At this stage, outer primers (LMB3 and fdseqlong) were added and additional 20 cycles were performed prior to a terminal elongation for 10 min at 72 °C. After assembly of sufficient amounts of full length randomized Fab constructs, they were digested *NcoI* / *NheI* and ligated into similarly treated acceptor phagemid vector. Purified ligations were used for ~60 transformations into electrocompetent *E. coli* TG1.
35 Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections. A final library size of 9.5×10^9 was obtained. Percentages

of functional clones, as determined by C-terminal tag detection in dot blot, were 81.1% for the light chain and 83.2% for the heavy chain, respectively.

Table 3 shows the sequence of generic phage-displayed antibody library (DP88-4), **Table 4** provides cDNA and amino acid sequences of library DP88-4 germline template and **Table 5** shows the Primer sequences used for generation of DP88-4 germline template.

Table 9 shows the sequence of generic phage-displayed lambda-DP47 library (VL3_19/VH3_23) template used for PCRs. **Table 10** provides cDNA and amino acid sequences of lambda-DP47 library (VL3_19/VH3_23) germline template and **Table 11** shows the Primer sequences used for generation of lambda-DP47 library (VL3_19/VH3_23).

Human, murine and cynomolgus 4-1BB (CD137) as antigens for the phage display selections and ELISA- and SPR-based screenings were transiently expressed as N-terminal monomeric Fc-fusion in HEK EBNA cells and in vivo site-specifically biotinylated via co-expression of BirA biotin ligase at the avi-tag recognition sequence located at the C-terminus of the Fc portion carrying the receptor chain (Fc knob chain).

Selection rounds (biopanning) were performed in solution according to the following procedure. First step, pre-clearing of $\sim 10^{12}$ phagemid particles on maxisorp plates coated with 10 $\mu\text{g/ml}$ of an unrelated human IgG to deplete the libraries of antibodies recognizing the Fc-portion of the antigen; second, incubation of the non-binding phagemid particles with 100 nM biotinylated human or murine 4-1BB for 0.5 h in the presence of 100 nM unrelated non-biotinylated Fc knob-into-hole construct for further depletion of Fc-binders in a total volume of 1ml; third, capture of biotinylated hu 4-1BB and attached specifically binding phage by transfer to 4 wells of a neutravidin pre-coated microtiter plate for 10 min (in rounds 1 & 3); fourth, washing of respective wells using 5x PBS/Tween20 and 5x PBS; fifth, elution of phage particles by addition of 250 μl 100mM TEA (triethylamine) per well for 10 min and neutralization by addition of 500 μl 1M Tris/HCl pH 7.4 to the pooled eluates from 4 wells; sixth, post-clearing of neutralized eluates by incubation on neutravidin pre-coated microtiter plate with 100nM biotin-captured Fc knob-into-hole construct for final removal of Fc-binders; seventh, re-infection of log-phase *E. coli* TG1 cells with the supernatant of eluted phage particles, infection with helperphage VCSM13, incubation on a shaker at 30°C over night and subsequent PEG/NaCl precipitation of phagemid particles to be used in the next selection round. Selections were carried out over 3 or 4 rounds using constant antigen concentrations of 100 nM. In rounds 2 and 4, in order to avoid enrichment of binders to neutravidin, capture of antigen: phage complexes was performed by addition of 5.4×10^7 streptavidin-coated magnetic beads. Specific binders were identified by ELISA as follows: 100 μl of 25 nM biotinylated human or murine 4-1BB and 10 $\mu\text{g/ml}$ of human IgG were coated on neutravidin plates and maxisorp plates, respectively. Fab-

containing bacterial supernatants were added and binding Fabs were detected via their Flag-tags using an anti-Flag/HRP secondary antibody. Clones exhibiting signals on human or murine 4-1BB and being negative on human IgG were short-listed for further analyses and were also tested in a similar fashion against the remaining two species of 4-1BB. They were bacterially expressed in a 0.5 liter culture volume, affinity purified and further characterized by SPR-analysis using BioRad's ProteOn XPR36 biosensor.

Clones 12B3, 25G7, 11D5 and 9B11 were identified as human 4-1BB-specific binder through the procedure described above. Clone 20G2 was identified as murine 4-1BB-specific binder through the procedure described above. The cDNA sequences of their variable regions are shown in **Table 15** below, the corresponding amino acid sequences can be found in **Table C**.

Table 15. Variable region base pair sequences for phage-derived anti-4-1BB antibodies. Underlined are the complementarity determining regions (CDRs).

Clone	SEQ ID NO:	Sequence
12B3	196 (VL)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTG TAGGAGACCGTGTCACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGG GGTCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGTATCATTCGTATCCGCAGACGTTTGGCC AGGGCACCAAAGTCGAGATCAAG
	197 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACCTACGCACAGAAGTTCCAGGGCAGGGTCACCATT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>TCTGAATTCCGTTTCTACGCTGACTTCGACTACTGGGGCCAAG</u> GGACCACCGTGACCGTCTCCTCA
25G7	198 (VL)	TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGG GACAGACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA <u>AGTTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCC</u> CCTGTACTTGTCTATGTTGTAACCAACCGGCCCTCAGGG ATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCT TCCTTGACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGAC TATTACTGTA <u>AACTCCCTTGATAGGCGCGGTATGTGGGTATTTCG</u> GCGGAGGGACCAAGCTGACCGTC

	199 (VH)	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCT GGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTACCT TTAGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGA AGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTA <u>GCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCT</u> CCAGAGACAATTCCAAGAACACGCTGTATCTGCAGATGAACA GCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGCGTG <u>ACGACCCGTGGCCGCCGTTCGACTACTGGGGCCAAGGAACCC</u> TGGTCACCGTCTCGAGT
11D5	200 (VL)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTG TAGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGG GGTCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGCTTAATTCGTATCCTCAGACGTTTGGCC AGGGCACCAAAGTCGAGATCAAG
	201 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACACTACGCACAGAAGTTCAGGGCAGGGTCACCATT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>TCTACTCTGATCTACGGTTACTTCGACTACTGGGGCCAAGGGA</u> CCACCGTGACCGTCTCCTCA
9B11	202 (VL)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTG TAGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGG GGTCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGGTTAATTCTTATCCGCAGACGTTTGGCC AGGGCACCAAAGTCGAGATCAAG
	203 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACACTACGCACAGAAGTTCAGGGCAGGGTCACCATT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>TCTTCTGGTGCTTACCCGGGTACTTCGACTACTGGGGCCAAG</u> GGACCACCGTGACCGTCTCCTCA

20G2	204 (VL)	GACATCCAGATGACCCAGTCTCCATCCACCCTGTCTGCATCTG TAGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTA <u>TTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAG</u> CCCCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGG GGTCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATT CACTCTCACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACT TATTACTGCCAACAGCAGCACTCGTATTATACGTTTGGCCAGG GCACCAAAGTCGAGATCAAG
	205 (VH)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCT GGGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACA TTCAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGA CAAGGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGT <u>ACAGCAAACCTACGCACAGAAAGTTCCAGGGCAGGGTCACCATT</u> ACTGCAGACAAATCCACGAGCACAGCCTACATGGAGCTGAGC AGCCTGAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGA <u>TCTTACTACTGGGAATCTTACCCGTTTCGACTACTGGGGCCAAG</u> GGACCACCGTGACCGTCTCCAGC

2.3 Preparation, purification and characterization of anti-4-1BB IgG1 P329G LALA antibodies

The variable regions of heavy and light chain DNA sequences of selected anti-4-1BB binders were subcloned in frame with either the constant heavy chain or the constant light chain of human IgG1. The Pro329Gly, Leu234Ala and Leu235Ala (i.e. P329G LALA) mutations have been introduced in the constant region of the knob and hole heavy chains to abrogate binding to Fc gamma receptors according to the method described in International Patent Appl. Publ. No. WO 2012/130831 A1.

The nucleotide and amino acid sequences of the anti-4-1BB clones are shown in **Table 16**. All anti-4-1BB-Fc-fusion encoding sequences were cloned into a plasmid vector, which drives expression of the insert from an MPSV promoter and contains a synthetic polyA signal sequence located at the 3' end of the CDS. In addition, the vector contains an EBV OriP sequence for episomal maintenance of the plasmid.

Table 16. Sequences of anti-4-1BB clones in P329GLALA human IgG1 format

Clone	SEQ ID No.	Sequence
12B3	206 (nucleotide sequence light chain)	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGT AGGAGACCGTGTCAACCATCACTTGCCGTGCCAGTCAGAGTATT AGTAGCTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAGCC CCTAAGCTCCTGATCTATGATGCCTCCAGTTTGGAAAGTGGGG TCCCATCACGTTTCAGCGGCAGTGGATCCGGGACAGAATTCAC TCTCACCATCAGCAGCTTGCAGCCTGATGATTTTGCAACTTATT ACTGCCAACAGTATCATTCGTATCCGCAGACGTTTGGCCAGGG CACCAAAGTCGAGATCAAGCGTACGGTGGCTGCACCATCTGTC

		TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGC CTCTGTTGTGTGCTGCTGAATAACTTCTATCCCAGAGAGGCCA AAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTC CCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTA CAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGA GAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTG AGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGT
	207 (nucleotide sequence heavy chain)	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTG GGTCCTCGGTGAAGGTCTCCTGCAAGGCCTCCGGAGGCACATT CAGCAGCTACGCTATAAGCTGGGTGCGACAGGCCCTGGACAA GGGCTCGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAG CAAACACGCACAGAAGTTCCAGGGCAGGGTCACCATTACTGC AGACAAATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCT GAGATCTGAGGACACCGCCGTGTATTACTGTGCGAGATCTGAA TTCCGTTTCTACGCTGACTTCGACTACTGGGGCCAAGGGACCA CCGTGACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTT CCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGACAGCG GCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGA CGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGCACAC CTTCCCGGTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCA GCGTGGTGACCGTGCCCTCCAGCAGCTTGGGACCCAGACCTA CATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGA CAAGAAAGTTGAGCCCAAATCTTGTGACAAAACCTCACACATGC CCACCGTGCCAGCACCTGAAGCTGCAGGGGGACCGTCAGTCT TCCTCTTCCCCCAAACCCAAAGGACACCCTCATGATCTCCCG GACCCCTGAGGTACATGCGTGGTGGTGGACGTGAGCCACGAA GACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGG TGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACA GCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGA CTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAA AGCCCTCGGCGCCCCATCGAGAAAACCATCTCCAAAGCCAAA GGGACGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCC GGGATGAGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGT CAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGC AATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCGTG CTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGT GGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCC GTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCC TCTCCCTGTCTCCGGGTAAA
	208 (Light chain)	DIQMTQSPSTLSASVGDRTITCRASQSISSWLAWYQQKPG KAPKLLIYDASSLESGVPSRFSGSGSGTEFTLTISLQPDFA TYYCQQYHSYPQTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ DSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSPVTK SFNRGEC
	209 (Heavy chain)	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAP GQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTAYME LSSLRSEDTAVYYCARSEFRFYADFDYWGQGTITVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPP KPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVVS

		NKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT CLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSGDSFFLY SKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
25G7	210 (nucleotide sequence light chain)	TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGG ACAGACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGAAG TTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCT GTACTTGTCTATCTATGGTAAAAACAACCGGCCCTCAGGGATCC CAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTT GACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGACTATTAC TGTAACCTCCCTTGATAGGCGCGGTATGTGGGTATTTCGGCGGAG GGACCAAGCTGACCGTCCTAGGTCAACCCAAGGCTGCCCCCAG CGTGACCCTGTTCCCCCCCCAGCAGCGAGGAAGTGCAGGCCAAC AAGGCCACCCTGGTCTGCCTGATCAGCGACTTCTACCCAGGCG CCGTGACCGTGCCCTGGAAGGCCGACAGCAGCCCCGTGAAGG CCGGCGTGAGACCACCACCCCCAGCAAGCAGAGCAACAACA AGTACGCCGCCAGCAGCTACCTGAGCCTGACCCCCGAGCAGTG GAAGAGCCACAGGTCTACAGCTGCCAGGTGACCCACGAGGG CAGCACCGTGAGAGAAAACCGTGCCCCCACCAGGTGCAGC
	211 (nucleotide sequence heavy chain)	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTG GGGGGTCCCTGAGACTCTCCTGTGCAGCCTCCGGATTACCTTT AGCAGTTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGG GGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCAC ATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGA GACAATTCCAAGAACACGCTGTATCTGCAGATGAACAGCCTGA GAGCCGAGGACACGGCCGTATATTACTGTGCGCGTGACGACCC GTGGCCGCCGTTTCGACTACTGGGGCCAAGGAACCCTGGTCACC GTCTCGAGTGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGG CACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGG CTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCTG TGGAACCTCAGGCGCCCTGACCAGCGGCGTGACACCTTCCCGG CTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTG ACCGTGCCCTCCAGCAGCTTGGGACCCAGACCTACATCTGCA ACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAG TTGAGCCCAAATCTTGTGACAAAACCTCACACATGCCACCGTG CCCAGCACCTGAAGCTGCAGGGGGACCGTCAGTCTTCTCTTC CCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTG AGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG AGGTCAAGTTCAACTGGTACGTGGACGGCGTGAGGTGCATAA TGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTA CCGTGTGGTCAGCGTCCTACCGTCCTGCACCAGGACTGGCTG AATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTC GGCGCCCCCATCGAGAAAACCATCTCCAAGGCCAAAGGGCAG CCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATG AGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG CAGCCGAGAAACAACCTACAAGACCACGCCTCCCGTGCTGGACT CCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAA GAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATG CATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCC TGTCTCCGGGTA
	212 (Light chain)	SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPV LVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSL DRRGMWVFGGGTKLTVLQPKAAPSVTLFPPSSEELQANKATLV CLISDFYPGAVTVAWKADSSPVKAGVETTPSKQSNKYYAASSYL

		SLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
	213 (Heavy chain)	EVQLLES G GGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKG LEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAE DTAVYYCARDDPWPPFDYWGQGLTVTVSSASTKGPSVFPLAPSS KSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSD GSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSP GK

The antibodies were produced by co-transfecting HEK293-EBNA cells with the mammalian expression vectors using polyethylenimine. The cells were transfected with the corresponding expression vectors in a 1:1 ratio ("vector heavy chain" : "vector light chain").

- 5 For production in 500 mL shake flasks, 400 million HEK293 EBNA cells were seeded 24 hours before transfection. For transfection cells were centrifuged for 5 minutes at 210 x g, and the supernatant was replaced by pre-warmed CD CHO medium. Expression vectors (200 µg of total DNA) were mixed in 20 mL CD CHO medium. After addition of 540 µL PEI, the solution was vortexed for 15 seconds and incubated for 10 minutes at room temperature. Afterwards,
- 10 cells were mixed with the DNA/PEI solution, transferred to a 500 mL shake flask and incubated for 3 hours at 37°C in an incubator with a 5% CO₂ atmosphere. After the incubation, 160 mL of F17 medium was added and cells were cultured for 24 hours. One day after transfection 1 mM valproic acid and 7% Feed with supplements were added. After culturing for 7 days, the supernatant was collected by centrifugation for 15 minutes at 210 x g. The solution was sterile
- 15 filtered (0.22 µm filter), supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4°C.

Purification of antibody molecules from cell culture supernatants was carried out by affinity chromatography using Protein A as described above for purification of antigen Fc fusions.

- 20 The protein was concentrated and filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 20mM Histidine, 140 mM NaCl solution of pH 6.0.

- The protein concentration of purified antibodies was determined by measuring the OD at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and molecular weight of the antibodies were analyzed by CE-SDS in the presence and
- 25 absence of a reducing agent (Invitrogen, USA) using a LabChipGXII (Caliper). The aggregate content of antibody samples was analyzed using a TSKgel G3000 SW XL analytical size-

exclusion column (Tosoh) equilibrated in a 25 mM K₂HPO₄, 125 mM NaCl, 200mM L-Arginine Monohydrochloride, 0.02 % (w/v) NaN₃, pH 6.7 running buffer at 25°C.

Table 17 summarizes the yield and final content of the anti-4-BB P329G LALA IgG1 antibodies.

5 **Table 17: Biochemical analysis of anti-4-BB P329G LALA IgG1 clones**

Clone	Yield [mg/l]	Monomer [%]	CE-SDS (non red)	CE-SDS (red)
12B3 P329GLALA IgG1	4	98	98.6% (173kDa)	22.5% (29kDa) 75.5% (64kDa)
25G7 P329GLALA IgG1	25	100	99.7% (181.6kDa)	76.8% (65kDa) 23% (42kDa)

In addition, an “untargeted” control DP47 huIgG1 P329G LALA antibody was prepared, in which the VH and VL of the 4-1BB binder was replaced by a germline control, termed DP47, which does not display binding to 4-1BB.

10

Example 3

Generation of bispecific antibodies targeting OX40 and fibroblast activation protein (FAP)

3.1 Generation of bispecific, trivalent antibodies targeting OX40 and fibroblast activation protein (FAP)

15 Bispecific agonistic OX40 constructs with trivalent binding for OX40, and trivalent binding for FAP (i.e. ‘3+3’ constructs) were prepared.

To enhance binding of Fab molecules to OX40, and for enhanced cross-linking of these receptors, trimerized Fab molecules targeting OX40 were generated by the method described in WO 2014/180754 A1.

20 Fab genes of the OX40 binders (VHCH1) were connected by a (GlySer)₂ linker to a short trimerisation domain derived from human cartilage matrix protein (CMP) (Uniprot Accession: P21941; Residues 454 to 496, SEQ ID NO:1) by standard recombinant DNA technologies. The cysteine residues forming interchain disulfide bridges at positions 458 and 460 were used together with the coiled coil domain comprising residues 467 to 495. Downstream of the CMP domain, a (Gly₄Ser)₄ linker was fused to connect the anti-FAP binding moiety to the
25 trimerisation domain. The FAP binding moieties were comprised of disulfide stabilized scFv fragments (H44/L100) of the FAP specific binder (28H1).

The CMP chains and anti-OX40 light chains were co-expressed, resulting in the production of bispecific, trimeric molecules as depicted in **Figure 2A**. The base pair and amino acid sequences for the constructs are shown in **Table 18** and **Table 19**, respectively.

Table 18. Base pair sequences of mature bispecific anti-OX40, anti-FAP CMP 3+3 format

Clone (OX40 clone/FAP clone)		SEQ ID NO.	Base pair sequence
3+3 (8H9/28H1)	Light chain (LC) (pCON184)	214	CATTCCGACATCCAGATGACCCAGTCTCCTTCC ACCCTGTCTGCATCTGTAGGAGACCGTGTCAACC ATCACTTGCCGTGCCAGTCAGAGTATTAGTAGC TGGTTGGCCTGGTATCAGCAGAAACCAGGGAAA GCCCCTAAGCTCCTGATCTATGATGCCTCCAGTT TGGAAAGTGGGGTCCCATCACGTTTCAGCGGCA GTGGATCCGGGACAGAATTCACCTCTCACCATCA GCAGCTTGCAGCCTGATGATTTTGCAACTTATTA CTGCCAACAGTATTTGACGTATTCGCGGTTTAC GTTTGGCCAGGGCACCAAAGTCGAGATCAAGCG TACGGTGGCTGCACCATCTGTCTTCATCTTCCCG CCATCTGATGAGCAGTTGAAATCTGGAAGTACC TCTGTTGTGTGCCTGCTGAATAACTTCTATCCCA GAGAGGCCAAAGTACAGTGGAAGGTGGATAAC GCCCTCCAATCGGGTAACTCCCAGGAGAGTGTC ACAGAGCAGGACAGCAAGGACAGCACCTACAG CCTCAGCAGCACCTGACGCTGAGCAAAGCAGA CTACGAGAAACACAAAGTCTACGCCTGCGAAGT CACCCATCAGGGCCTGAGCTCGCCCGTCACAAA GAGCTTCAACAGGGGAGAGTGT

	CMP chain pETR12239	215	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTG AAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGC AAGGCCTCCGGAGGACATTACAGCAGCTACGCT ATAAGCTGGGTGCGACAGGCCCCTGGACAAGG GCTCGAGTGGATGGGAGGGATCATCCCTATCTT TGGTACAGCAAACACTACGCACAGAAGTTCCAGGG CAGGGTCACCATTACTGCAGACAAATCCACGAG CACAGCCTACATGGAGCTGAGCAGCCTGAGATC TGAGGACACCGCCGTGTATTACTGTGCGAGAGA ATACGGTTGGATGGACTACTGGGGCCAAGGGAC CACCGTGACCGTCTCCTCAGCTAGCACAAAGGG ACCTAGCGTGTTCCCCCTGGCCCCCAGCAGCAA GTCTACATCTGGCGGAACAGCCGCCCTGGGCTG CCTCGTGAAGGACTACTTTCCCGAGCCCGTGAC CGTGTCTGGAACCTCTGGCGCTCTGACAAGCGG CGTGCACACCTTTCCAGCCGTGCTGCAGAGCAG CGGCCTGTACTCTCTGAGCAGCGTCGTGACAGT GCCCAGCAGCTCTCTGGGCACCCAGACCTACAT CTGCAACGTGAACCACAAGCCCAGCAACACCA AGGTGGACAAGAAGGTGGAACCCAAGAGCTGC GACGGCGGAGGGGGATCTGGCGGCGGAGGATC CGAGGAAGATCCTTGCGCCTGCGAGAGCCTCGT GAAGTTCCAGGCCAAGGTGGAAGGACTGCTGC AGGCCCTGACCCGGAAACTGGAAGCCGTGTCCA AGCGGCTGGCCATCCTGGAAAACACCGTGGTGT CCGGAGGCGGGGGTAGCGGCGGAGGGGGCTCT GGCGGTGGCGGGTCTGGAGGCGGGGGTTCAGA AGTGCAGCTGCTGGAATCTGGCGGCGGACTGGT GCAGCCTGGCGGATCTCTGAGACTGAGCTGTGC CGCCAGCGGCTTCACCTTTAGCAGCCACGCCAT GAGCTGGGTGCGCCAGGCCCCTGGAAAGTGCCT GGAATGGGTGTCCGCCATCTGGGCCAGCGGCGA GCAGTACTACGCCGATAGCGTGAAGGGCCGGTT CACCATCAGCCGGGACAACAGCAAGAACACCC TGTACCTGCAGATGAACAGCCTGCGGGCCGAGG ACACCGCCGTGTACTATTGTGCCAAGGGCTGGC TGGGCAACTTCGACTATTGGGGCCAGGGCACCC TCGTGACCGTGTCTAGCGGAGGGGGCGGAAGTG GTGGCGGGGGAAGCGGCGGGGGTGGCAGCGGA GGGGGCGGATCTGAAATTGTGCTGACCCAGAGC CCTGGCACCCCTGAGCCTGTCTCCAGGCGAAAGA GCCACACTGAGCTGCAGAGCCAGCCAGAGCGT GTCCAGAAGCTACCTGGCCTGGTATCAGCAGAA GCCCCGACAGGCCCCCAGACTGCTGATCATCGG CGCCTCTACAAGAGCCACCGGCATCCCCGATAG ATTCAGCGGCTCTGGCAGCGGCACCGACTTCAC CCTGACCATCAGCAGACTGGAACCCGAGGACTT TGCCGTGTATTACTGCCAGCAGGGCCAAGTGAT CCCCCCCACCTTTGGCTGTGGCACAAAGGTGGA AATCAAA
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3+3 (1G4/28H1)	LC (pCON251)	216	CATTTCGGACATCCAGATGACCCAGTCTCCTTCC ACCCTGTCTGCATCTGTAGGAGACCGTGTCAAC ATCACTTGCCGTGCCAGTCAGAGTATTAGTAGC TGGTTGGCCTGGTATCAGCAGAAACCAGGGAAA GCCCCTAAGCTCCTGATCTATGATGCCTCCAGTT TGGAAAGTGGGGTCCCATCACGTTTCAGCGGCA GTGGATCCGGGACAGAATTCACCTCTCACCATCA GCAGCTTGCAGCCTGATGATTTTGCAACTTATTA CTGCCAACAGTATATTTTCGTATTCCATGTTGACG TTTGGCCAGGGCACCAAAGTCGAGATCAAGCGT ACGGTGGCTGCACCATCTGTCTTCATCTTCCCGC CATCTGATGAGCAGTTGAAATCTGGAAGTGCCT CTGTTGTGTGCCTGCTGAATAACTTCTATCCCAG AGAGGCCAAAGTACAGTGGAAGGTGGATAACG CCCTCCAATCGGGTAACTCCCAGGAGAGTGTCA CAGAGCAGGACAGCAAGGACAGCACCTACAGC CTCAGCAGCACCTGACGCTGAGCAAAGCAGAC TACGAGAAACACAAAGTCTACGCCTGCGAAGTC ACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG AGCTTCAACAGGGGAGAGTGT
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	CMP chain (pETR12640)	217	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTG AAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGC AAGGCCTCCGGAGGACATTACAGCAGCTACGCT ATAAGCTGGGTGCGACAGGCCCCTGGACAAGG GCTCGAGTGGATGGGAGGGATCATCCCTATCTT TGGTACAGCAAACACTACGCACAGAAGTTCCAGGG CAGGGTCACCATTACTGCAGACAAATCCACGAG CACAGCCTACATGGAGCTGAGCAGCCTGAGATC TGAGGACACCGCCGTGTATTACTGTGCGAGAGA ATACGGTTCTATGGACTACTGGGGCCAAGGGAC CACCGTGACCGTCTCCTCAGCTAGCACAAAGGG ACCTAGCGTGTTCCCCCTGGCCCCCAGCAGCAA GTCTACATCTGGCGGAACAGCCGCCCTGGGCTG CCTCGTGAAGGACTACTTTCCCGAGCCCGTGAC CGTGTCTGGAACCTCTGGCGCTCTGACAAGCGG CGTGCACACCTTTCCAGCCGTGCTGCAGAGCAG CGGCCTGTACTCTCTGAGCAGCGTCGTGACAGT GCCCAGCAGCTCTCTGGGCACCCAGACCTACAT CTGCAACGTGAACCACAAGCCCAGCAACACCA AGGTGGACAAGAAGGTGGAACCCAAGAGCTGC GACGGCGGAGGGGGATCTGGCGGCGGAGGATC CGAGGAAGATCCTTGCGCCTGCGAGAGCCTCGT GAAGTTCCAGGCCAAGGTGGAAGGACTGCTGC AGGCCCTGACCCGGAAACTGGAAGCCGTGTCCA AGCGGCTGGCCATCCTGGAACACCGTGGTGT CCGGAGGCGGGGGTAGCGGCGGAGGGGGCTCT GGCGGTGGCGGGTCTGGAGGCGGGGGTTCAGA AGTGCAGCTGCTGGAATCTGGCGGCGGACTGGT GCAGCCTGGCGGATCTCTGAGACTGAGCTGTGC CGCCAGCGGCTTCACCTTTAGCAGCCACGCCAT GAGCTGGGTGCGCCAGGCCCCTGGAAAGTGCCT GGAATGGGTGTCCGCCATCTGGGCCAGCGGCGA GCAGTACTACGCCGATAGCGTGAAGGGCCGGTT CACCATCAGCCGGGACAACAGCAAGAACACCC TGTACCTGCAGATGAACAGCCTGCGGGCCGAGG ACACCGCCGTGTACTATTGTGCCAAGGGCTGGC TGGGCAACTTCGACTATTGGGGCCAGGGCACCC TCGTGACCGTGTCTAGCGGAGGGGGCGGAAGTG GTGGCGGGGGAAGCGGCGGGGGTGGCAGCGGA GGGGGCGGATCTGAAATTGTGCTGACCCAGAGC CCTGGCACCCCTGAGCCTGTCTCCAGGCGAAAGA GCCACACTGAGCTGCAGAGCCAGCCAGAGCGT GTCCAGAAGCTACCTGGCCTGGTATCAGCAGAA GCCCCGACAGGCCCCCAGACTGCTGATCATCGG CGCCTCTACAAGAGCCACCGGCATCCCCGATAG ATTCAGCGGCTCTGGCAGCGGCACCGACTTCAC CCTGACCATCAGCAGACTGGAACCCGAGGACTT TGCCGTGTATTACTGCCAGCAGGGCCAAGTGAT CCCCCCCACCTTTGGCTGTGGCACAAAGGTGGA AATCAAA
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3+3 (49B4/28H1)	LC (pCON323)	218	GACATCCAGATGACCCAGTCTCCTTCCACCCTG TCTGCATCTGTAGGAGACCGTGTCAACCATCACT TGCCGTGCCAGTCAGAGTATTAGTAGCTGGTTG GCCTGGTATCAGCAGAAACCAGGGAAAGCCCCT AAGCTCCTGATCTATGATGCCTCCAGTTTGGAA AGTGGGGTCCCATCACGTTTCAGCGGCAGTGGA TCCGGGACAGAATTCACTCTCACCATCAGCAGC TTGCAGCCTGATGATTTTGCAACTTATTACTGCC AACAGTATAGTTTCGCAGCCGTATACGTTTGGCC AGGGCACCAAAGTCGAGATCAAGCGTACGGTG GCTGCACCATCTGTCTTCATCTTCCCGCCATCTG ATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTG TGTGCCTGCTGAATAACTTCTATCCCAGAGAGG CCAAAGTACAGTGGAAGGTGGATAACGCCCTCC AATCGGGTAACTCCCAGGAGAGTGTACAGAGC AGGACAGCAAGGACAGCACCTACAGCCTCAGC AGCACCTGACGCTGAGCAAAGCAGACTACGA GAAACACAAAGTCTACGCCTGCGAAGTCACCCA TCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT CAACAGGGGAGAGTGT
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	CMP chain (pETR12899)	219	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTG AAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGC AAGGCCTCCGGAGGCACATTCAGCAGCTACGCT ATAAGCTGGGTGCGACAGGCCCTGGACAAGG GCTCGAGTGGATGGGAGGGATCATCCCTATCTT TGGTACAGCAAACCTACGCACAGAAGTTCCAGGG CAGGGTCACCATTACTGCAGACAAATCCACGAG CACAGCCTACATGGAGCTGAGCAGCCTGAGATC TGAGGACACCGCCGTGTATTACTGTGCGAGAGA ATACTACCGTGGTCCGTACGACTACTGGGGCCA AGGGACCACCGTGACCGTCTCCTCAGCTAGCAC AAAGGGACCTAGCGTGTTCCCCCTGGCCCCCAG CAGCAAGTCTACATCTGGCGGAACAGCCGCCCT GGGCTGCCTCGTGAAGGACTACTTTCCCGAGCC CGTGACCGTGTCTGGAACCTCTGGCGCTCTGAC AAGCGGCGTGACACCTTTCCAGCCGTGCTGCA GAGCAGCGGCCTGTACTCTCTGAGCAGCGTCGT GACAGTGCCAGCAGCTCTCTGGGCACCCAGAC CTACATCTGCAACGTGAACCACAAGCCCAGCAA CACCAAGGTGGACAAGAAGGTGGAACCCAAGA GCTGCGACGGCGGAGGGGGATCTGGCGGCGGA GGATCCGAGGAAGATCCTTGCGCCTGCGAGAGC CTCGTGAAGTTCCAGGCCAAGGTGGAAGGACTG CTGCAGGCCCTGACCCGGAACCTGGAAGCCGTG TCCAAGCGGCTGGCCATCCTGGAAAACACCGTG GTGTCCGGAGGCGGGGGTAGCGGCGGAGGGGG CTCTGGCGGTGGCGGGTCTGGAGGCGGGGGTTC AGAAGTGCAGCTGCTGGAATCTGGCGGCGGACT GGTGCAGCCTGGCGGATCTCTGAGACTGAGCTG TGCCGCCAGCGGCTTCACCTTTAGCAGCCACGC CATGAGCTGGGTGCGCCAGGCCCTGGAAAGTG CCTGGAATGGGTGTCCGCCATCTGGGCCAGCGG CGAGCAGTACTACGCCGATAGCGTGAAGGGCC GGTTCACCATCAGCCGGGACAACAGCAAGAAC ACCCTGTACCTGCAGATGAACAGCCTGCGGGCC GAGGACACCGCCGTGTACTATTGTGCCAAGGGC TGGCTGGGCAACTTCGACTATTGGGGCCAGGGC ACCCTCGTGACCGTGTCTAGCGGAGGGGGCGGA AGTGGTGGCGGGGGAAGCGGCGGGGGTGGCAG CGGAGGGGGCGGATCTGAAATTGTGCTGACCCA GAGCCCTGGCACCCCTGAGCCTGTCTCCAGGCGA AAGAGCCACACTGAGCTGCAGAGCCAGCCAGA GCGTGTCCAGAAGCTACCTGGCCTGGTATCAGC AGAAGCCCGGACAGGCCCCAGACTGCTGATCA TCGGCGCCTCTACAAGAGCCACCGGCATCCCCG ATAGATTCAGCGGCTCTGGCAGCGGCACCGACT TCACCCTGACCATCAGCAGACTGGAACCCGAGG ACTTTGCCGTGTATTACTGCCAGCAGGGCCAAG TGATCCCCCCCACCTTTGGCTGTGGCACAAAGG TGGAATCAAA
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3+3 (21H4/28H 1)	LC (pETR12026)	220	GACATCCAGATGACCCAGTCTCCTTCCACCCTG TCTGCATCTGTAGGAGACCGTGTCAACCATCACT TGCCGTGCCAGTCAGAGTATTAGTAGCTGGTTG GCCTGGTATCAGCAGAAACCAGGGAAAGCCCCT AAGCTCCTGATCTATGATGCCTCCAGTTTGGAA AGTGGGGTCCCATCACGTTTCAGCGGCAGTGGA TCCGGGACAGAATTCACTCTCACCATCAGCAGC TTGCAGCCTGATGATTTTGCAACTTATTACTGCC AACAGTATCAGACGTATCCTGTTACGTTTGGCC AGGGCACCAAAGTCGAGATCAAGCGTACGGTG GCTGCACCATCTGTCTTCATCTTCCCGCCATCTG ATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTG TGTGCCTGCTGAATAACTTCTATCCCAGAGAGG CCAAAGTACAGTGGAAGGTGGATAACGCCCTCC AATCGGGTAACTCCCAGGAGAGTGTACAGAGC AGGACAGCAAGGACAGCACCTACAGCCTCAGC AGCACCTGACGCTGAGCAAAGCAGACTACGA GAAACACAAAGTCTACGCCTGCGAAGTCACCCA TCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT CAACAGGGGAGAGTGT
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	CMP chain (pETR12810)	221	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTG AAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGC AAGGCCTCCGGAGGACATTCAGCAGCTACGCT ATAAGCTGGGTGCGACAGGCCCTGGACAAGG GCTCGAGTGGATGGGAGGGATCATCCCTATCTT TGGTACAGCAAACACTACGCACAGAAGTTCCAGGG CAGGGTCACCATTACTGCAGACAAATCCACGAG CACAGCCTACATGGAGCTGAGCAGCCTGAGATC TGAGGACACCGCCGTGTATTACTGTGCGAGAGA CCCGCGTGGTCCGTACTTCCCGTACTTCGACTAC TGGGGCCAAGGGACCACCGTGACCGTCTCCTCA GCTAGCACAAAGGGACCTAGCGTGTTCCTCCCTG GCCCCCAGCAGCAAGTCTACATCTGGCGGAACA GCCGCCCTGGGCTGCCTCGTGAAGGACTACTTT CCCGAGCCCGTGACCGTGTCTGGAAGTCTGGC GCTCTGACAAGCGGCGTGCACACCTTCCAGCC GTGCTGCAGAGCAGCGGCCTGTACTCTCTGAGC AGCGTCGTGACAGTGCCAGCAGCTCTCTGGGC ACCCAGACCTACATCTGCAACGTGAACCACAAG CCCAGCAACACCAAGGTGGACAAGAAGGTGGA ACCCAAGAGCTGCGACGGCGGAGGGGGATCTG GCGGCGGAGGATCCGAGGAAGATCCTTGCGCCT GCGAGAGCCTCGTGAAGTTCCAGGCCAAGGTGG AAGGACTGCTGCAGGCCCTGACCCGAAACTGG AAGCCGTGTCCAAGCGGCTGGCCATCCTGAAA ACACCGTGGTGTCCGGAGGCGGGGTAGCGGC GGAGGGGGCTCTGGCGGTGGCGGGTCTGGAGG CGGGGGTTCAGAAGTGCAGCTGCTGGAATCTGG CGGCGGACTGGTGCAGCCTGGCGGATCTCTGAG ACTGAGCTGTGCCGCCAGCGGCTTCACCTTTAG CAGCCACGCCATGAGCTGGGTGCGCCAGGCCCC TGGAAGTGCCTGGAATGGGTGTCCGCCATCTG GGCCAGCGGCGAGCAGTACTACGCCGATAGCGT GAAGGGCCGGTTCACCATCAGCCGGGACAACA GCAAGAACACCCTGTACCTGCAGATGAACAGCC TGCGGGCCGAGGACACCGCCGTGTACTATTGTG CCAAGGGCTGGCTGGGCAACTTCGACTATTGGG GCCAGGGCACCCCTCGTGACCGTGTCTAGCGGAG GGGCGGAAGTGGTGGCGGGGGAAGCGGCGGG GGTGGCAGCGGAGGGGGCGGATCTGAAATTGT GCTGACCCAGAGCCCTGGCACCCCTGAGCCTGTC TCCAGGCGAAAGAGCCACACTGAGCTGCAGAG CCAGCCAGAGCGTGTCCAGAAGCTACCTGGCCT GGTATCAGCAGAAGCCCGGACAGGCCCCCAGA CTGCTGATCATCGGCGCCTCTACAAGAGCCACC GGCATCCCCGATAGATTCAGCGGCTCTGGCAGC GGCACCGACTTCACCCTGACCATCAGCAGACTG GAACCCGAGGACTTTGCCGTGTATTACTGCCAG CAGGGCCAAGTGATCCCCCCCACCTTTGGCTGT GGCACAAAGGTGGAAATCAAA
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3+3 (CLC563 /28H1)	LC (pCON586)	222	GAGATCGTGCTGACCCAGAGCCCCGGCACACTC TCCCTGTCTCCTGGGGAAAGGGCCACCCTTTCA TGCAGAGCCAGCCAGTCCGTCTCTAGTAGCTAC CTGGCATGGTATCAGCAGAAGCCAGGACAAGC CCCCGCCTCCTGATTTACGGCGCTTCCTCTCGG GCAACTGGTATCCCTGACAGGTTCTCAGGGAGC GGAAGCGGAACAGATTTTACCTTGACTATTTCT AGACTGGAGCCAGAGGACTTCGCCGTGTATTAC TGTCAGCAGTACGGTAGTAGCCCCCTCACCTTT GGCCAGGGGACAAAAGTCGAAATCAAGCGTAC GGTGGCTGCACCATCTGTCTTCATCTTCCCGCCA TCTGATGAGCAGTTGAAATCTGGAAGTGCCTCT GTTGTGTGCCTGCTGAATAACTTCTATCCCAGA GAGGCCAAAGTACAGTGGAAGGTGGATAACGC CCTCCAATCGGGTAACTCCCAGGAGAGTGTAC AGAGCAGGACAGCAAGGACAGCACCTACAGCC TCAGCAGCACCTGACGCTGAGCAAAGCAGACT ACGAGAAACACAAAGTCTACGCCTGCGAAGTC ACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG AGCTTCAACAGGGGAGAGTGT
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	CMP chain (pETR12898)	223	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTG GTACAGCCTGGGGGGTCCCTGAGACTCTCCTGT GCAGCCTCCGGATTCACCTTTAGCAGTTATGCC ATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGG GCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGG TGGTAGCACATACTACGCAGACTCCGTGAAGGG CCGGTTCACCATCTCCAGAGACAATTCCAAGAA CACGCTGTATCTGCAGATGAACAGCCTGAGAGC CGAGGACACGGCCGTATATTACTGTGCGCTTGA CGTTGGTGCTTTCGACTACTGGGGCCAAGGAGC CCTGGTCACCGTCTCGAGTGCTAGCACAAAGGG ACCTAGCGTGTTCCCCCTGGCCCCCAGCAGCAA GTCTACATCTGGCGGAACAGCCGCCCTGGGCTG CCTCGTGAAGGACTACTTTCCCGAGCCCGTGAC CGTGTCTGGAACCTCTGGCGCTCTGACAAGCGG CGTGCACACCTTTCCAGCCGTGCTGCAGAGCAG CGGCCTGTACTCTCTGAGCAGCGTCGTGACAGT GCCCAGCAGCTCTCTGGGCACCCAGACCTACAT CTGCAACGTGAACCACAAGCCCAGCAACACCA AGGTGGACAAGAAGGTGGAACCCAAGAGCTGC GACGGCGGAGGGGGATCTGGCGGCGGAGGATC CGAGGAAGATCCTTGCGCCTGCGAGAGCCTCGT GAAGTTCCAGGCCAAGGTGGAAGGACTGCTGC AGGCCCTGACCCGGAAACTGGAAGCCGTGTCCA AGCGGCTGGCCATCCTGGAAAACACCGTGGTGT CCGGAGGCGGGGGTAGCGGCGGAGGGGGCTCT GGCGGTGGCGGGTCTGGAGGCGGGGGTTCAGA AGTGCAGCTGCTGGAATCTGGCGGCGGACTGGT GCAGCCTGGCGGATCTCTGAGACTGAGCTGTGC CGCCAGCGGCTTCACCTTTAGCAGCCACGCCAT GAGCTGGGTGCGCCAGGCCCTGGAAAGTGCCT GGAATGGGTGTCCGCCATCTGGGCCAGCGGCGA GCAGTACTACGCCGATAGCGTGAAGGGCCGGTT CACCATCAGCCGGGACAACAGCAAGAACACCC TGTACCTGCAGATGAACAGCCTGCGGGCCGAGG ACACCGCCGTGTACTATTGTGCCAAGGGCTGGC TGGGCAACTTCGACTATTGGGGCCAGGGCACCC TCGTGACCGTGTCTAGCGGAGGGGGCGGAAGTG GTGGCGGGGGAAGCGGCGGGGGTGGCAGCGGA GGGGGCGGATCTGAAATTGTGCTGACCCAGAGC CCTGGCACCCCTGAGCCTGTCTCCAGGCGAAAGA GCCACACTGAGCTGCAGAGCCAGCCAGAGCGT GTCCAGAAGCTACCTGGCCTGGTATCAGCAGAA GCCCCGACAGGCCCCCAGACTGCTGATCATCGG CGCCTCTACAAGAGCCACCGGCATCCCCGATAG ATTCAGCGGCTCTGGCAGCGGCACCGACTTCAC CCTGACCATCAGCAGACTGGAACCCGAGGACTT TGCCGTGTATTACTGCCAGCAGGGCCAAGTGAT CCCCCCCACCTTTGGCTGTGGCACAAAGGTGGA AATCAAA
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3+3 (20B7 /28H1)	LC (pCON260)	224	GACATCCAGATGACCCAGTCTCCTTCCACCCTG TCTGCATCTGTAGGAGACCGTGTCAACCATCACT TGCCGTGCCAGTCAGAGTATTAGTAGCTGGTTG GCCTGGTATCAGCAGAAACCAGGGAAAGCCCCT AAGCTCCTGATCTATGATGCCTCCAGTTTGGAA AGTGGGGTCCCATCACGTTTCAGCGGCAGTGGA TCCGGGACAGAATTCACTCTCACCATCAGCAGC TTGCAGCCTGATGATTTTGCAACTTATTACTGCC AACAGTATCAGGCTTTTTTCGCTTACGTTTGGCCA GGGCACCAAAGTCGAGATCAAGCGTACGGTGG CTGCACCATCTGTCTTCATCTTCCCGCCATCTGA TGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGT GTGCCTGCTGAATAACTTCTATCCCAGAGAGGC CAAAGTACAGTGGAAGGTGGATAACGCCCTCCA ATCGGGTAACTCCCAGGAGAGTGTACACAGAGCA GGACAGCAAGGACAGCACCTACAGCCTCAGCA GCACCCTGACGCTGAGCAAAGCAGACTACGAG AAACACAAAGTCTACGCCTGCGAAGTCACCCAT CAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTC AACAGGGGAGAGTGT
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	CMP chain (pETR12639)	225	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTG AAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGC AAGGCCTCCGGAGGACATTCAGCAGCTACGCT ATAAGCTGGGTGCGACAGGCCCCTGGACAAGG GCTCGAGTGGATGGGAGGGATCATCCCTATCTT TGGTACAGCAAACCTACGCACAGAAGTTCCAGGG CAGGGTCACCATTACTGCAGACAAATCCACGAG CACAGCCTACATGGAGCTGAGCAGCCTGAGATC TGAGGACACCGCCGTGTATTACTGTGCGAGAGT TAACTACCCGTACTCTTACTGGGGTGACTTCGA CTACTGGGGCCAAGGGACCACCGTGACCGTCTC CTCAGCTAGCACAAAGGGACCTAGCGTGTTCCC CCTGGCCCCCAGCAGCAAGTCTACATCTGGCGG AACAGCCGCCCTGGGCTGCCTCGTGAAGGACTA CTTTCCCGAGCCCGTGACCGTGTCTGGAACCTCT GGCGCTCTGACAAGCGGCGTGACACCTTTCCA GCCGTGCTGCAGAGCAGCGGCCTGTACTCTCTG AGCAGCGTCTGTGACAGTGCCAGCAGCTCTCTG GGCACCAGACCTACATCTGCAACGTGAACCAC AAGCCCAGCAACACCAAGGTGGACAAGAAGGT GGAACCCAAGAGCTGCGACGGCGGAGGGGGAT CTGGCGGCGGAGGATCCGAGGAAGATCCTTGCG CCTGCGAGAGCCTCGTGAAGTTCCAGGCCAAGG TGGAAGGACTGCTGCAGGCCCTGACCCGGAAC TGGAAGCCGTGTCCAAGCGGCTGGCCATCCTGG AAAACACCGTGGTGTCCGGAGGCGGGGGTAGC GGCGGAGGGGGCTCTGGCGGTGGCGGGTCTGG AGGCGGGGGTTCAGAAGTGCAGCTGCTGGAATC TGGCGGCGGACTGGTGCAGCCTGGCGGATCTCT GAGACTGAGCTGTGCCGCCAGCGGCTTCACCTT TAGCAGCCACGCCATGAGCTGGGTGCGCCAGGC CCCTGGAAAGTGCCTGGAATGGGTGTCCGCCAT CTGGGCCAGCGGCGAGCAGTACTACGCCGATAG CGTGAAGGGCCGGTTCACCATCAGCCGGGACAA CAGCAAGAACACCCTGTACCTGCAGATGAACAG CCTGCGGGCCGAGGACACCGCCGTGTACTATTG TGCCAAGGGCTGGCTGGGCAACTTCGACTATTG GGGCCAGGGCACCCCTCGTGACCGTGTCTAGCGG AGGGGGCGGAAGTGGTGGCGGGGGAAGCGGCG GGGGTGGCAGCGGAGGGGGCGGATCTGAAATT GTGCTGACCCAGAGCCCTGGCACCCCTGAGCCTG TCTCCAGGCGAAAGAGCCACACTGAGCTGCAGA GCCAGCCAGAGCGTGTCCAGAAGCTACCTGGCC TGGTATCAGCAGAAGCCCGGACAGGCCCCCAG ACTGCTGATCATCGGCGCCTCTACAAGAGCCAC CGGCATCCCCGATAGATTGAGCGGCTCTGGCAG CGGCACCGACTTCACCCTGACCATCAGCAGACT GGAACCCGAGGACTTTGCCGTGTATTACTGCCA GCAGGGCCAAGTGATCCCCCCCACCTTTGGCTG TGGCACAAAGGTGGAAATCAAA
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Table 19. Amino acid sequence of mature bispecific anti-OX40, anti-FAP CMP 3+3 format

Clone		SEQ ID NO.	Amino acid sequence
3+3 (8H9/28H1)	Light chain (LC) (pCON184)	226	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPGKAPKLLIYDASSLESGVPSRFSGSGS GTEFTLTISLQPDDEFATYYCQQYLTYSRFTFG QGTEKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESV TEQDSKDSSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC
	CMP chain pETR12239	227	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQAPGQGLEWMGGIPIFGTANYAQKF QGRVTITADKSTSTAYMELSSLRSEDATVYYC AREYGWMDYWGQGTTVTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDGGGG SGGGGSEEDPCACESLVKFKQAKVEGLLQALTR KLEAVSKRLAILENTVVSGGGGSGGGGSGGGG SGGGGSEVQLLESGLLVQPGGSLRLSCAASG FTFSSHAMSWVRQAPGKCLEWVSATWASGEQ YYADSVKGRFTISRDNKNTLYLQMNSLRAD TAVYYCAKRWLGDFDYWGQGLTVTVSSGGG GSGGGGSGGGGSGGGGSEIVLTQSPGTLSSLSPG ERATLSCRASQSVSRSLAWYQQKPGQAPRLL IIGASTRATGIPDRFSGSGSGTDFTLTISRLEPED FAVYYCQQGQVIPPTFGCGTKVEIK
3+3 (1G4/28H1)	pCON251	228	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPGKAPKLLIYDASSLESGVPSRFSGSGS GTEFTLTISLQPDDEFATYYCQQYISYSMLTFG QGTEKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESV TEQDSKDSSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC

	CMP chain (pETR12640)	229	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQAPGQGLEWMGGIPIFGTANYAQKF QGRVTITADKSTSTAYMELSSLRSED TAVYYC AREYGSMDYWGQGTTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TQTYICNVNHKPSNTKVDKKVEPKSCDGGGGS GGGGSEEDPCACESLVKFQAKVEGLLQALTRK LEAVSKRLAILENTVVSGGGGSGGGGSGGGGS GGGGSEVQLLES GGGGLVQPGGSLRLSCAASGF TFSSHAMSWVRQAPGKCLEWVSAIWASGEQY YADSVKGRFTISRDN SKNTLYLQMNSLRAEDT AVYYCAKGWLG NFDYWGQGT LVTVSSGGGG SGGGGSGGGGSGGGGSEIVLTQSPGTL SLSPGE RATLSCRASQS VSRSYLAWYQQKPGQAPRLLI GASTRATGIPDRFSGSGSGTDFTLTISRLEPEDF AVYYCQQGQVIPPTFGCGTKVEIK
	LC (pCON323)	230	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPGKAPKLLIYDASSLESGVPSRFSGSGS GTEFTLTISSLQPDDFATYYCQQYSSQPYTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVC LLNFPYPREAKVQWKVDNALQSGNSQESVTE QDSKDSTYSLSTLTLSKADYEEKHKVYACEVT HQGLSSPVTKSFNRGEC
3+3 (49B4/28H1)	CMP chain (pETR12899)	231	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQAPGQGLEWMGGIPIFGTANYAQKF QGRVTITADKSTSTAYMELSSLRSED TAVYYC AREYYRGPYDYWGQGTTVTVSSASTKGPSVFP LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDGGGG SGGGGSEEDPCACESLVKFQAKVEGLLQALTR KLEAVSKRLAILENTVVSGGGGSGGGGSGGGG SGGGGSEVQLLES GGGGLVQPGGSLRLSCAASG FTSSHAMSWVRQAPGKCLEWVSAIWASGEQ YYADSVKGRFTISRDN SKNTLYLQMNSLRAED TAVYYCAKGWLG NFDYWGQGT LVTVSSGGG GSGGGGSGGGGSGGGGSEIVLTQSPGTL SLSPG ERATLSCRASQS VSRSYLAWYQQKPGQAPRLL IIGASTRATGIPDRFSGSGSGTDFTLTISRLEPED FAVYYCQQGQVIPPTFGCGTKVEIK

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3+3 (21H4/28H 1)	LC (pETR1202 6)	232	DIQMTQSPSTLSASVGDRVITICRASQSISSWLA WYQQKPGKAPKLLIYDASSLESGVPSRFSGSGS GTEFTLTISSLQPDDFATYYCQQYQTYPTVTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVC LLNNFYPREAKVQWKVDNALQSGNSQESVTE QDSKIDSTYLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC
	CMP chain (pETR1281 0)	233	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQAPGQGLEWMGGIPIFGTANYAQKF QGRVTITADKSTSTAYMELSSLRSEDTAVYYC ARDPRGPYPYFDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SSLGTQTYICNVNHKPSNTKVDKKVEPKSCDG GGGSGGGGSEEDPCACESLVKFQAKVEGLLQA LTRKLEAVSKRLAILENTVVSAGGGGSGGGGSG GGGSGGGGSEVQLLESAGGGLVQPGGSLRLSCA ASGFTFSSHAMSWVRQAPGKCLEWVSIAIWA GEQYYADSVKGRFTISRDN SKNTLYLQMNSLR AEDTAVYYCAKGWLG NFDYWGQGT LTVTVSS GGGSGGGGSGGGGSGGGGSGGGGSEIVLTQSPGTL LSPGERATLSCRASQSVSRSYLA WYQQKPGQA PRLLIIGASTRATGIPDRFSGSGSGTDFTLTISR LEPEDFAVYYCQQGQVIPPTFGCGTKVEIK
3+3 (CLC563 /28H1)	LC (pCON586)	234	EIVLTQSPGTLSPGERATLSCRASQSVSSSYL AWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG SGTDFTLTISRLEPEDFAVYYCQQYGSSPLTFG QGKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESV TEQDSKIDSTYLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC

	CMP chain (pETR1289 8)	235	EVQLLESGGGLVQPGGSLRLSCAASGFTSSYA MSWVRQAPGKGLEWVSAISGSGGSTYYADSV KGRFTISRDN SKNTLYLQMNSLRAEDTAVYYC ALDVGA FDYWGQ GALVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TQTYICNVNHKPSNTKVDKKVEPKSCDGGGGS GGGGSEEDPCACESLVKFQAKVEGLLQALTRK LEAVSKRLAILENTVVSGGGGSGGGGSGGGGS GGGGSEVQLLES GGGLVQPGGSLRLSCAASGF TFSSHAMSWVRQAPGKCLEWVSAIWASGEQY YADSVKGRFTISRDN SKNTLYLQMNSLRAEDT AVYYCAKGWLG NFDYWGQ GTLVTVSSGGGG SGGGGSGGGGSGGGGSEIVLTQSPGTL SLSPGE RATLSCRASQSVSRSYLAWYQQKPGQAPRLLII GASTRATGIPDRFSGSGSGTDFTLTISRLEPEDF AVYYCQQGQVIPPTFGCGTKVEIK
	LC (pCON260)	236	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLA WYQQKPGKAPKLLIYDASSLESGVPSRFSGSGS GTEFTLTISSLQPDDFATYYCQYQAFSLTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVC LLNFPYPREAKVQWKVDNALQSGNSQESVTE QDSKDSTYSLSTLTLSKADYEEKHKVYACEVT HQGLSSPVTKSFNRGEC
3+3 (20B7 /28H1)	CMP chain (pETR1263 9)	237	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQAPGQGLEWMGGIPIFGTANYAQKF QGRVTITADKSTSTAYMELSSLRSEDTAVYYC ARVNYPYSYWGDFDYWGQGTTVTVSSASTKG PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC DGGGGSGGGGSEEDPCACESLVKFQAKVEGLL QALTRKLEAVSKRLAILENTVVSGGGGSGGGG SGGGGSGGGGSEVQLLES GGGLVQPGGSLRLS CAASGFTFSSHAMSWVRQAPGKCLEWVSAIW ASGEQYYADSVKGRFTISRDN SKNTLYLQMNS LRAEDTAVYYCAKGWLG NFDYWGQ GTLVTV SSGGGGSGGGGSGGGGSGGGGSEIVLTQSPGT LSLSPGERATLSCRASQSVSRSYLAWYQQKPG QAPRLLIIGASTRATGIPDRFSGSGSGTDFTLTIS RLEPEDFAVYYCQQGQVIPPTFGCGTKVEIK

All genes were transiently expressed under the control of a chimeric MPSV promoter, consisting of the MPSV core promoter combined with the CMV promoter enhancer fragment.

The used expression vectors also contain the oriP region for episomal replication in Epstein Barr Virus Nuclear Antigen (EBNA) containing host cells.

For production of the trimeric, bispecific anti-OX40, anti-FAP molecules constructs were produced by co-transfecting HEK293-EBNA cells with the mammalian expression vectors using polyethylenimine. The cells were transfected with the corresponding expression vectors in a 1:1 ratio ("CMP vector" : "LC").

For production in 500 mL shake flasks, 250 million HEK293 EBNA cells were seeded 24 hours before transfection in Excell media with supplements. For transfection, the cells were centrifuged for 5 minutes at 210 x g, and supernatant was replaced by pre-warmed CD-CHO medium. Expression vectors were mixed in 20 mL CD-CHO medium to a final amount of 200 µg DNA. After addition of 540 µL PEI (1 mg/mL), the solution was vortexed for 15 seconds and incubated for 10 minutes at room temperature. Subsequently, cells were mixed with the DNA/PEI solution, transferred to a 500 mL shake flask and incubated for 3 hours at 37°C in an incubator with a 5% CO₂ atmosphere, with shaking at 165 rpm. After the incubation, 160 mL Excell medium with supplements was added and cells were cultured for 24 hours. At this point the valproic acid concentration was 1 mM (the media also contained 5 g/L PepSoy and 6 mM L-Glutamine). 24h after transfection the cells were supplemented with Feed 7 at 12% final volume (24 mL) and 3 g/L glucose (1.2 mL from 500 g/L stock). After culturing for 7 days, the cell supernatant was collected by centrifugation for 45 minutes at 2000-3000 x g. The solution was sterile filtered (0.22 µm filter), supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4°C.

Purification of bispecific constructs from cell culture supernatants was carried out by affinity chromatography using CH1 kappa select material (BAC), followed by size exclusion chromatography. The protein was concentrated and filtered prior to loading on the HiLoad Superdex 200 column (GE Healthcare) equilibrated with 20mM Histidine, 140mM NaCl, 0.01% Tween-20 solution of pH 6.0.

For affinity chromatography, the supernatant was loaded on a CH1 kappa select column (Life Sciences) (Column Volume = 4.5 mL) equilibrated with 36 mL 50 mM Tris-HCl, 100 mM Glycine, 150 mM sodium chloride, pH 8.0. Unbound protein was removed by washing with 10 column volumes of 50 mM Tris-HCl, 150 mM Glycine, 150 mM sodium chloride, pH 8.0. The bound protein was eluted using a linear pH-gradient of 20 CVs from 0 to 100% of 50 mM Tris-

HCl, 100 mM Glycine, 150 mM sodium chloride, pH 2.0. The column was then washed with 10 column volumes of 50 mM Tris-HCl, 100 mM Glycine, 150 mM sodium chloride, pH 2.0 followed by a re-equilibration step.

The pH of the collected fractions was adjusted by adding 1/10 (v/v) of 2M Tris-HCl, pH 8.0. The protein was concentrated and filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 20 mM Histidine, 140 mM NaCl, pH 6.0, 0.01% Tween20.

The protein concentration of purified bispecific constructs was determined by measuring the OD at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and molecular weight of the bispecific constructs were analyzed by CE-SDS in the presence and absence of a reducing agent (Invitrogen, USA) using a LabChipGXII (Caliper). The aggregate content of bispecific constructs was analyzed using a TSKgel G3000 SW XL analytical size-exclusion column (Tosoh) equilibrated in a 25 mM K₂HPO₄, 125 mM NaCl, 200mM L-Arginine Monohydrochloride, 0.02 % (w/v) NaN₃, pH 6.7 running buffer at 25°C (Table 20).

Table 20. Biochemical analysis of bispecific anti-OX40, anti-FAP CMP 3+3 constructs

Clone	Yield [mg/l]	Monomer [%]	CE-SDS (non red)	CE-SDS (red)
OX40 8H9/FAP 28H1 3+3 (ID4971)	1.2	99.7	0.94% (179 kDa) 2.14% (204 kDa) 2.34% (222 kDa) 94.57% (239 kDa)	17.84% (28 kDa) 2.06% (29kDa) 1.66% (31 kDa) 78.44% (65 kDa)
OX40 1G4/FAP 28H1 3+3 (ID5185)	5.5	96.6	0.21% (210 kDa) 0.36% (227 kDa) 99.43% (244 kDa)	24.56% (30 kDa) 0.72% (32 kDa) 2.96% (62 kDa) 71.76% (66 kDa)

OX40 49B4/FAP 28H1 3+3 (ID5350)	6.5	93	0.31% (32 kDa) 0.29% (56 kDa) 0.32% (170 kDa) 0.12% (183 kDa) 0.38% (224 kDa) 98.58% (239 kDa)	0.2% (26 kDa) 25.92% (29 kDa) 0.55% (32 kDa) 0.27% (37 kDa) 0.24% (50 kDa) 2.05% (60 kDa) 70.47% (66 kDa)
OX40 21H4/FAP 28H1 3+3 (ID5278)	11.2	98	0.35% (204 kDa) 0.54% (220 kDa) 99.12% (233 kDa)	25.85% (29 kDa) 2.94% (33 kDa) 6.76% (92 kDa) 62.95% (67 kDa) Some minor peaks < 0.5%
OX40 CLC563/FAP 28H1 3+3 (ID5351)	8.7	98.2	0.24% (30 kDa) 0.3% (53 kDa) 0.29% (201 kDa) 0.5% (219 kDa) 98.66% (235 kDa)	25.52% (27 kDa) 0.42% (29 kDa) 0.79% (60 kDa) 73.27% (64 kDa)
OX40 20B7/FAP 28H1 3+3 (ID5235)	0.17	96.9	1.94% (32 kDa) 7.2% (57 kDa) 0.27% (177 kDa) 19.52% (230 kDa) 71.06% (236 kDa)	26.33% (30 kDa) 2.08% (32 kDa) 4.18% (62 kDa) 66.82% (66 kDa) 0.29% (96 kDa) 0.29% (122 kDa)

3.2 Determination of the aggregation temperature of bispecific trivalent 3+3 constructs

For direct comparison of all formats the thermal stability was monitored by Static Light
 5 Scattering (SLS) and by measuring the intrinsic protein fluorescence in response to applied
 temperature stress. 30 mg of filtered protein sample with a protein concentration of 1 mg/ml was
 applied in duplicate to an Optim 2 instrument (Avacta Analytical Ltd). The temperature was
 ramped from 25 to 85 °C at 0.1 °C/min, with the radius and total scattering intensity being
 collected. For determination of intrinsic protein fluorescence the sample was excited at 266 nm
 10 and emission was collected between 275 nm and 460 nm.

Table 21. Aggregation temperatures for the bispecific, trivalent anti-OX40, anti-FAP CMP 3+3 constructs

Construct	8H9/28H1 3+3	1G4/28H1 3+3	49B4/28H1 3+3	21H4/28H1 3+3	CLC563/28H1 3+3	20B7/28H1 3+3
Tagg (°C)	54	56	53	56	61	nd

Example 4

Binding of trimeric, bispecific antigen binding molecules targeting OX40 and FAP

15 4.1 Analysis of simultaneous binding to OX40 and FAP by surface plasmon resonance analysis

The ability of the trimeric, bispecific antibodies to bind simultaneously to human OX40
 Fc(kih) and human FAP was assessed by surface plasmon resonance (SPR) analysis. All SPR
 experiments were performed on a Biacore T200 at 25 °C with HBS-EP as running buffer (0.01
 20 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, Biacore,
 Freiburg/Germany).

Biotinylated human OX40 Fc(kih) was directly coupled to the chip surface of a
 streptavidin (SA) sensor chip. Immobilization levels up to 1000 resonance units (RU) were used.

The bispecific constructs targeting OX40 and FAP were passed over the chip surface at a
 25 concentration of 250 nM, and at a flow rate of 30 µL/minute for 90 seconds, with a dissociation
 phase set to zero sec. Human FAP was injected as the second analyte, at a concentration of 250

nM, and at a flow rate of 30 μ L/minute for 90 seconds (**Figure 3A**). Dissociation was monitored for 120 sec. Bulk refractive index differences were corrected for by subtracting the response obtained in a reference flow cell, where no protein was immobilized.

All of the trimeric, bispecific anti-OX40, anti-FAP molecules were found to be able to
5 bind simultaneously to human OX40 and human FAP (**Figure 4A to 4E**).

4.2 Binding to human OX40 expressing cells: analysis of binding to naïve and activated human peripheral mononuclear blood mononuclear cells (PBMCs)

Human PBMCs were isolated from buffy coat of healthy donors obtained from the blood
donation center Zurich (Blutspendezentrum Schlieren, Switzerland) by Ficoll density gradient
10 centrifugation. Buffy coats were diluted with an equal volume of DPBS (Gibco by Life
Technologies, Cat. No. 14190 326). 50 mL polypropylene centrifuge tubes (TPP, Cat.-No. 91050)
were supplied with 15 mL Histopaque 1077 (SIGMA Life Science, Cat.-No. 10771, polysucrose
and sodium diatrizoate, adjusted to a density of 1.077 g/mL) and the buffy coat solution was
layered above the Histopaque 1077. The tubes were centrifuged for 30 min at 400 x g at room
15 temperature, and with low acceleration and no break. Subsequently the PBMCs were collected
from the interphase, washed three times with DPBS and resuspended in T cell medium
consisting of RPMI 1640 medium (Gibco by Life Technology, Cat. No. 42401-042)
supplemented with 10 % Fetal Bovine Serum (FBS, Gibco by Life Technology, Cat. No. 16000-
044, Lot 941273, gamma-irradiated, mycoplasma-free and heat inactivated at 56 °C for 35 min),
20 1 % (v/v) GlutaMAX I (GIBCO by Life Technologies, Cat. No. 35050 038), 1 mM Sodium-
Pyruvate (SIGMA, Cat. No. S8636), 1 % (v/v) MEM non-essential amino acids (SIGMA, Cat.-No.
M7145) and 50 μ M β -Mercaptoethanol (SIGMA, M3148).

PBMCs were used in experiments either directly after isolation (for analysis of binding to
resting human PBMCs), or following stimulation to provide for high expression human OX40 on
25 the cell surface of T cells (for analysis of binding to activated human PBMCs). For stimulations,
naïve PBMCs were cultured for three days in T cell medium supplied with 200 U/mL Proleukin
and 2 μ g/mL PHA-L, in 6-well tissue culture plates, and subsequently cultured for 1 day on pre-
coated 6-well tissue culture plates (pre-coated with 10 μ g/mL anti-human CD3 (clone OKT3)
and 2 μ g/mL anti-human CD28 (clone CD28.2)) in T cell medium supplied with 200 U/mL
30 Proleukin, at 37 °C and 5% CO₂.

For detection of OX40, naïve human PBMCs and activated human PBMCs were mixed.
To enable distinction of the naïve cells from the activated human PBMCs, resting cells were
labeled prior to the binding assay using the eFluor670 cell proliferation dye (eBioscience, Cat.-
No.65-0840-85).

For labeling, cells were harvested, washed with pre-warmed (37°C) DPBS and adjusted to a cell density of 1×10^7 cells/mL in DPBS. eFluor670 cell proliferation dye (eBioscience, Cat.-No.65-0840-85) was added to the suspension of naïve human PBMCs at a final concentration of 2.5 mM, and a final cell density of 0.5×10^7 cells/mL in DPBS. Cells were then incubated for 10 min at room temperature in the dark. To stop the labeling reaction, 2 mL FBS was added and cells were washed three times with T cell medium. A 1:1 mixture of 1×10^5 naïve, eFluor670-labeled human PBMC and unlabeled, activated human PBMC was then added to wells of round-bottomed suspension cell 96-well plates (Greiner bio-one, Cellstar, Cat. No. 650185).

Plates were centrifuged 4 minutes at $400 \times g$ at 4 °C, and the supernatant was removed. Cells were washed once with 200 µL, 4°C FACS buffer (DPBS supplemented with 2 % FBS, 5 mM EDTA pH 8 (Amresco, Cat. No. E177) and 7.5 mM sodium azide (Sigma-Aldrich S2002)). Cells were resuspended in 50 µL/well of 4 °C cold FACS buffer containing titrated bispecific anti-OX40, anti-FAP molecules for 120 minutes at 4 °C. Plates were then washed four times with 200 µL/well 4 °C FACS buffer to remove unbound molecules.

Cells were subsequently stained for 30 minutes at 4°C in the dark in 25 µL/well, 4 °C FACS buffer containing fluorescently-labeled anti-human CD4 (clone RPA-T4, mouse IgG1 k, BioLegend, Cat.-No. 300532), anti-human CD8 (clone RPa-T8, mouse IgG1k, BioLegend, Cat.-No. 3010441), anti-human CD45 (clone HI30, mouse IgG1k, BioLegend, Cat.-No. 304028), and Fluorescein isothiocyanate (FITC)-conjugated AffiniPure anti-human IgG F(ab')₂-fragment-specific goat IgG F(ab')₂ fragment (Jackson ImmunoResearch, Cat. No. 109-096-097).

Cells were then washed twice with 200 µL/well 4 °C FACS buffer, resuspended in 80 µL/well FACS-buffer containing 0.2 µg/mL DAPI (Santa Cruz Biotech, Cat. No. Sc-3598) and acquired the same day using 5-laser LSR-Fortessa (BD Bioscience with DIVA software).

As shown in **Figures 5A to 5D**, none of the trimeric, bispecific anti-OX40, anti-FAP molecules bound to resting human CD4⁺ T cells or CD8⁺ T cells, which do not express OX40. By contrast, all of the trimeric, bispecific anti-OX40, anti-FAP molecules displayed binding to activated CD8⁺ or CD4⁺ T cells, which express OX40. Binding to activated CD4⁺ T-cells was much stronger than binding to activated CD8⁺ T cells. Activated human CD8⁺ T cells express OX40 at a much lower level than the level of expression by activated CD4⁺ T cells. Donor- and time-dependent differences in binding were observed.

The molecules were also found to differ in the strength of binding. EC₅₀ values are shown in **Table 22**. For further evaluation of bivalent and monovalent FAP targeted constructs clones with high (8H9) and low (49B4/1G4) binding capacity were chosen.

Table 22. EC₅₀ values of binding to activated human CD4 T cells

Clone	EC ₅₀ [nM]
8H9	1.01
CLC563	1.93
49B4	1.40
21H4	4.57
1G4	0.39

4.3 Binding to human FAP-expressing and FAP-negative tumor cells

Binding of the trimeric, bispecific anti-OX40, anti-FAP molecules to FAP expressed at the cell surface was analysed using the FAP-expressing human melanoma cell line WM266-4 cells (ATCC CRL-1676), and using the FAP-negative ovarian hamster tumor cell line CHO/dhFr- (ATCC CRL-9096) as a negative control.

In some of the experiments CHO/dhFr- cells were pre-labeled with PKH-26 Red Fluorescence Cell linker Kit (Sigma, Cat.-No. PKH26GL) to enable separation from unlabeled tumor cells. CHO/dhFr- cells were harvested and washed three times with RPMI 1640 medium. The cells were then stained for 5 minutes at room temperature in the dark at a final cell density of 1×10^7 cells in freshly prepared PKH26-Red-stain solution (at a final concentration of 1 nM, in diluent C (provided with the PKH-26 Red Fluorescence Cell linker Kit)). Excess FBS was added to stop labeling reaction, and cells were then washed four times with RPMI 1640 medium supplemented with 10 % (v/v) FBS, 1 % (v/v) GlutaMAX-I, to remove excess dye.

0.5×10^5 PKH26-labeled CHO/dhFr- and unlabeled WM266-4 cells were then added to wells of round-bottomed suspension cell 96-well plates (Greiner Bio-one, Cellstar, Cat. No. 650185) and the binding assay was performed in a similar manner as described in **Example 4.2** hereinabove. Plates were centrifuged 4 minutes at $400 \times g$ at 4°C , and the supernatant was removed. Cells were washed once with 200 μL , 4°C FACS buffer (DPBS supplemented with 2 % FBS, 5 mM EDTA pH 8 (Amresco, Cat. No. E177) and 7.5 mM sodium azide (Sigma-Aldrich S2002)). Cells were resuspended in 50 μL /well of 4°C cold FACS buffer containing titrated bispecific anti-OX40, anti-FAP molecules for 120 minutes at 4°C . Plates were then washed four times with 200 μL /well 4°C FACS buffer to remove unbound molecules. Cells were further stained with 25 μL /well of 4°C secondary antibody solution containing Fluorescein isothiocyanate (FITC)-conjugated AffiniPure anti-human IgG F(ab')₂-fragment-specific goat IgG F(ab')₂ fragment (Jackson ImmunoResearch, Cat. No. 109-096-097), and incubated for 30 minutes at 4°C in the dark. Cells were then resuspended in 80 μL /well FACS-buffer containing

0.2 µg/mL DAPI (Santa Cruz Biotech, Cat. No. Sc-3598) and acquired the same day using 5-laser LSR-Fortessa (BD Bioscience with DIVA software).

As shown in **Figures 6A** and **6B**, the trimeric, bispecific anti-OX40, anti-FAP molecules bound efficiently to human FAP-expressing WM266-4 cells, but not to FAP-negative CHO/dhFr- cells. Therefore, bispecific, trimeric anti-FAP and anti-OX40 antigen binding molecules show direct tumor-targeting properties. Differences in the ability of the molecules to bind to cell surface FAP were observed, which might be caused by sterical hindrance with the molecules. EC₅₀ values of binding to FAP positive tumor cells are summarized in **Table 23**.

Table 23. EC₅₀ values for binding of selected aOX40 binder in a FAP targeted trivalent format to cell surface human FAP

Clone	EC ₅₀ [nM]
8H9	1.36
CLC563	2.16
49B4	2.15
21H4	5.03
1G4	0.29

Example 5

Biological Activity of trimeric, bispecific antigen binding molecules targeting OX40 and FAP

5.1 HeLa cells expressing human OX40 and reporter gene NF-κB-luciferase

Agonistic binding of OX40 to its ligand induces downstream signaling via activation of nuclear factor kappa B (NFκB) (A. D. Weinberg et al., J. Leukoc. Biol. 2004, 75(6), 962-972). The recombinant reporter cell line HeLa_hOX40_NFκB_Luc1 expressing human OX40 on its surface was generated. This cell line harbors a reporter plasmid containing the luciferase gene under the control of an NFκB-sensitive enhancer segment. Binding and activation of OX40 induces dose-dependent activation of NFκB, which then translocates to the nucleus, where it binds to the NFκB-sensitive enhancer of the reporter plasmid to increase expression of the luciferase protein. Luciferase catalyzes luciferin-oxidation resulting in oxyluciferin, which emits light. This can be detected and quantified using a luminometer.

Adherent HeLa_hOX40_NFκB_Luc1 cells were harvested using cell dissociation buffer (Invitrogen, Cat.-No. 13151-014) for 10 minutes at 37 °C. Cells were washed once with DPBS

and were adjusted to a cell density of 2×10^5 in assay media comprising of MEM (Invitrogen, Cat.-No. 22561-021), 10 % (v/v) heat-inactivated FBS, 1 mM Sodium-Pyruvate and 1% (v/v) non-essential amino acids. Cells were seeded in a density of 0.3×10^5 cells per well in a sterile, white 96-well-flat bottomed tissue culture plate with lid (greiner bio-one, Cat. No. 655083) and incubated overnight at 37 °C in a 5% CO₂ atmosphere, in an incubator (Hera Cell 150).

The next day, HeLa_hOX40_NFκB_Luc1 cells were stimulated for 6 hours by adding assay medium containing various titrated antigen binding molecules targeting OX40 and FAP. To analyse the effect of hyper-crosslinking by cell surface FAP binding, 25 μL/well of medium containing FAP⁺ tumor cells (NIH/3T3-huFAP clone 39) were co-cultured with HeLa_hOX40_NFκB_Luc1 cells at a ratio of 2:1 (i.e. there were twice as many FAP⁺ tumor cells as reporter cells, per well). NIH/3T3-huFAP clone 39 was generated by the transfection of the mouse embryonic fibroblast NIH/3T3 cell line (ATCC CRL-1658) with the expression vector pETR4921 to express human FAP under a CMV promoter, under puromycin selection (1.5 μg/mL; InvivoGen, Cat.-No.: ant-pr-5).

After incubation, supernatant was aspirated and plates were washed two times with DPBS. Quantification of light emission was analysed using the luciferase 1000 assay system and the reporter lysis buffer (both Promega, Cat.-No. E4550 and Cat-No: E3971), according to the manufacturer's instructions. Briefly, cells were lysed for 10 minutes at -20 °C by addition of 30 μL per well 1x lysis buffer. Cells were thawed for 20 minutes at 37 °C before 90 μL of luciferase assay reagent was added per well. Light emission was quantified immediately with a SpectraMax M5/M5e microplate reader (Molecular Devices, USA) using 500ms integration time, without any filter to collect all wavelengths. Emitted units of released light (URL) were corrected using readings for basal luminescence of HeLa_hOX40_NFκB_Luc1 cells, and were plotted against the logarithmic primary antibody concentration using Prism4 (GraphPad Software, USA). Where possible, trend lines were fitted to the data using the inbuilt sigmoidal dose response.

As shown in **Figures 7A to 7E**, a limited, dose-dependent activation of NFκB was observed to be induced by addition of the trivalent FAP-targeted, anti-OX40 clones 49B4, 1G4, CLC-563, 21H4 and 8H9 to the HeLa_hOX40_NFκB_Luc1 reporter cells (dotted lines, open triangle). This is in line with the observation that the trimeric OX40L naturally engages three OX40 receptors on the cell surface to form the basic signaling unit capable of recruiting TRAFs to the cytosolic part of the OX40 receptor, initiating NFκB activation (Vinay DS et al. 2009. Cell Biol Int. 33(4):453-65). Hyper-crosslinking of the antigen binding molecules bound to the reported cells by FAP-expressing NIH/3T3huFAP strongly increased the induction of NFκB-mediated luciferase-activation, in a concentration-dependent manner (solid lines, filled circle). It has been speculated that full agonism of the OX40 axis can only be achieved when at least a hexameric OX40 signaling unit is assembled (Vinay DS et al. 2009. Cell Biol Int. 33(4):453-65),

which could be facilitated by FAP-mediated immobilization of the FAP targeted trivalent molecules. However, with increasing concentration a drop in bioactivity was observed after an initial increase. It is thought that at high concentrations of the antigen binding molecule, efficient hypercrosslinking is limited by insufficient FAP numbers at the cell surface, or by steric hindrance. NF κ B activation drops to the levels observed for addition of trimeric molecules without hypercrosslinking.

The EC₅₀ values of NF κ B activation in the absence of hypercrosslinking are summarized in **Table 24**. The bioactivity in the absence of hypercrosslinking (**Figure 8B**) was subtracted from the bioactivity in the presence of FAP positive cells (**Figure 8A**) for each evaluated concentration, and this was then plotted against the concentration of the antigen binding molecule (**Figure 8C**) to provide an estimate of the increase in bioactivity achieved by targeting the trivalent constructs to FAP. This can be best appreciated when the agonistic capacity of each construct was quantified as the area under the curve (AUC) and plotted against each other (**Figure 8D**).

Table 24. EC₅₀ values of NF κ B activation in the HeLa_hOX40_NF κ B_luc1 reporter cell line co-incubated with bispecific, trimeric anti-FAP, anti-OX40 antigen binding molecules.

Clone	EC ₅₀ [nM]
8H9	0.33
CLC563	0.56
49B4	1.01
1G4	0.10
21H4	4.38

5.2 OX40 mediated costimulation of suboptimally TCR triggered pre-activated human CD4 T cells

Ligation of OX40 provides a synergistic co-stimulatory signal promoting division and survival of T-cells following suboptimal T-cell receptor (TCR) stimulation (M. Croft et al., Immunol. Rev. 2009, 229(1), 173-191). Additionally, production of several cytokines and surface expression of T-cell activation markers is increased following ligation of OX40 (I. Gramaglia et al., J. Immunol. 1998, 161(12), 6510-6517; S. M. Jensen et al., Seminars in Oncology 2010, 37(5), 524-532).

To analyse the agonistic properties of the bispecific, trivalent, anti-FAP and anti-OX40 antigen binding molecules, pre-activated OX40-positive CD4⁺ T-cells were stimulated for 72

hours with a suboptimal concentration of plate-immobilized anti-CD3 antibodies, in the presence of anti-OX40 antigen binding molecules immobilized on the plate surface. The effects on T-cell survival and proliferation were analyzed by monitoring of total cell counts and CFSE dilution in living cells by flow cytometry. Additionally, cells were co-stained with fluorescently-labeled antibodies against T-cell activation and differentiation markers, e.g. CD127 and TIM-3.

Human PBMCs were isolated via ficoll density centrifugation and were stimulated for three days with PHA-L (2 $\mu\text{g/mL}$) and Proleukin (200 U/mL) as described in **Example 4.2** hereinabove. Cells were then labeled with CFSE at a cell density of 1×10^6 cells/mL with CFDA-SE (Sigma-Aldrich, Cat.-No. 2188), at a final concentration of 50 nM, for 10 minutes at 37 °C. Thereafter, cells were washed twice with excess DPBS containing FBS (10% v/v). Labeled cells were rested in T-cell media at 37 °C for 30 minutes. Thereafter, non-converted CFDA-SE was removed by two additional washing steps with DPBS. CD4⁺ T-cell isolation from pre-activated CFSE-labeled human PBMCs was performed using the MACS negative CD4 T-cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions.

Morris et al. previously showed that agonistic co-stimulation with conventional anti-OX40 antibodies relied on surface immobilization (N. P. Morris et al., Mol. Immunol. 2007, 44(12), 3112-3121). Thus, goat anti-mouse Fc γ -specific antibodies (Jackson ImmunoResearch, Ca.No. 111-500-5008) and goat anti-human Fab-specific antibodies (Sigma, Cat.-No. I5260) were coated to the surface of a 96 well U-bottomed cell culture plates (Greiner Bio One) at a concentration of 2 $\mu\text{g/mL}$ in PBS overnight at 4 °C. Thereafter, the plate surface was blocked with DPBS containing BSA (1 % v/w). All following incubation steps were done at 37 °C for 90 minutes in PBS containing BSA (1 % v/w). Between the incubation steps, plates were washed with DPBS. Mouse anti-human CD3 antibody (clone OKT3, eBioscience, Ca.No. 16-0037-85, fixed concentration of 3 ng/mL) was captured in a subsequent incubation step via the surface coated anti-mouse Fc γ -specific antibodies. The FAP-targeted trivalent anti-OX40 molecules were then immobilized on plate by an additional incubation step in DPBS, via binding to the plate-coated goat anti-human Fab-specific antibodies.

CFSE-labeled preactivated CD4⁺ T cells were added to the pre-coated plates at a cell density of 1×10^5 cells per well in 200 μL T-cell media, and cultured for 96 hours. Cells were stained with a combination of fluorochrome-labeled mouse anti-human TIM-3 (clone F38-2E2, Biolegend, Ca.No.345008) and anti-CD127 (clone A019D5, Biolegend, Ca.No.351234) for 20 minutes at 4 °C in the dark. Plates were washed twice with 200 μL /well 4 °C FACS buffer, were finally resuspended in 80 μL /well FACS-buffer containing 0.2 $\mu\text{g/mL}$ DAPI (Santa Cruz Biotech, Cat. No. Sc-3598) and acquired the same day using 5-laser LSR-Fortessa (BD Bioscience with DIVA software).

Co-stimulation with plate-immobilized trivalent anti-OX40 antigen binding molecules strongly enhanced suboptimal stimulation of pre-activated human CD4 T cells with plate-immobilized anti-human CD3, in a dose dependent manner (**Figures 9A to 9D**). T-cells proliferated more strongly, and displayed a more mature phenotype as indicated by a higher percentage of CD127 low T cells, and a higher percentage of TIM-3 positive activated cells. Half-maximal changes in all analyzed parameters of T-cell activation were achieved at concentrations ranging from 10 to 3000 pM, and are summarized in **Figure 10**.

5.3 OX40 mediated costimulation of suboptimally TCR triggered resting human PBMC and hypercrosslinking by cell surface FAP

It was shown in **Example 5.1** that addition of FAP⁺ tumor cells can strongly increase the NFκB activity induced by FAP-targeted, trivalent anti-OX40 molecules in a human OX40-positive reporter cell line, by providing strong oligomerization of OX40 receptors. These constructs were therefore evaluated in the presence of NIH/3T3-huFAP clone 39 cells for their ability to rescue suboptimal TCR stimulation of resting human PBMC cells.

Human PBMC preparations contain (1) resting OX40-negative CD4⁺ and CD8⁺ T cells and (2) antigen presenting cells with various Fc-γ receptor molecules on their cell surface e.g. B cells and monocytes. Anti-human CD3 antibody of human IgG1 isotype can bind with its Fc part to the present Fc-γ receptor molecules and mediate a prolonged TCR activation on resting OX40 negative CD4⁺ and CD8⁺ T cells. These cells then start to express OX40 within several hours.

Functional agonistic compounds against OX40 can signal via the OX40 receptor present on activated CD8⁺ and CD4⁺ T cells and support TCR-mediated stimulation.

Resting CFSE-labeled human PBMCs were stimulated for four days with a suboptimal concentration of anti-CD3 antibody in the presence of irradiated FAP⁺ NIH/3T3-huFAP clone 39 cells and titrated anti-OX40 constructs. The effects on T-cell survival and proliferation were analyzed through monitoring of total cell counts and CFSE dilution in living cells by flow cytometry. Additionally, cells were co-stained with fluorescently-labeled antibodies against T-cell activation marker CD25.

Mouse embryonic fibroblast NIH/3T3-huFAP clone 39 cells (**Example 5.1**) were harvested using cell dissociation buffer (Invitrogen, Cat.-No. 13151-014) for 10 minutes at 37 °C. Cells were washed once with DPBS. NIH/3T3-huFAP clone 39 cells were cultured at a density of 0.2×10^5 cells per well in T cell media in a sterile 96-well round bottomed adhesion tissue culture plate (TPP, Cat. No. 92097) overnight at 37 °C and 5% CO₂ in an incubator (Hera Cell 150). The next day they were irradiated in an xRay irradiator using a dose of 45 Gy to prevent later overgrowth of human PBMC by the tumor cell line.

Human PBMCs were isolated by ficoll density centrifugation and were labeled with CFSE as described in **Example 4.2**. Cells were added to each well at a density of 0.75×10^5 cells per well. Anti-human CD3 antibody (clone V9, human IgG1) at a final concentration of 2 nM, and FAP targeted trivalent OX40 antigen binding molecules or non-targeted trivalent OX40 antigen binding molecules were added at the indicated concentrations. Cells were activated for four days at 37 °C and 5% CO₂ in an incubator (Hera Cell 150). Cells were subsequently surface-stained with fluorescent dye-conjugated antibodies anti-human CD4 (clone RPA-T4, BioLegend, Cat.-No. 300532), CD8 (clone RPa-T8, BioLegend, Cat.-No. 3010441) and CD25 (clone M-A251, BioLegend, Cat.-No. 356112) for 20 min at 4 °C. For permeabilizing the cell membrane, cell pellets were washed twice with FACS buffer, then resuspended in 50 µL/well freshly prepared FoxP3 Fix/Perm buffer (eBioscience, Cat.-No. 00-5123 and 00-5223) for 45 min at room temperature in the dark. After washing the cells three times with Perm-Wash buffer (eBioscience, Cat.-No. 00-8333-56), cells were stained intracellular with 25 µL/well Perm-Wash Buffer containing anti-human Granzyme B antibody (clone GB-11, BD Bioscience, Cat. No. 561142) for 1 h at room temperature in the dark. Cells were then resuspended in 85 µL/well FACS buffer, and acquired using a 5-laser Fortessa flow cytometer (BD Bioscience with DIVA software).

As shown in **Figures 11A to 11D**, costimulation with non-targeted trivalent anti-OX40 barely rescued suboptimally TCR-stimulated CD4 T cells (as determined by analysis for CD25 expression). Hyper-crosslinking of the FAP-targeted trivalent anti-OX40 constructs by the NIH/3T3-huFAP clone 39 cells strongly promoted proliferation, survival and induced an enhanced activated phenotype in human CD4 and CD8 T cells (as indicated by CD25 expression). High affinity clone 8H9 (**Figures 11A and 11B**), showed again a peak activity at ~0.1–1 nM, and a reduced response at higher concentration. Similar to the findings in the NFκB reporter cell line (**Figure 7D**, as described in **Example 5.1**) this might be a consequence of competition for target binding between constructs that bind only to FAP or OX40, and those that bind simultaneously to both targets and provide thus the necessary cross-linking function. This effect was not seen when trivalent constructs were coated to plates. The bell shaped effect was less prominent for all other clones in the FAP-targeted, trivalent anti-OX40 format (**Figure 11**).

5.4 Relationship between binding strength to OX40 and bioactivity of trimeric, bispecific antigen binding molecules targeting OX40 and FAP molecules

Figure 12 shows the correlation between the strength of binding to cells, and the strength of NFκB activation (as determined as AUC for NFκB activation; **Figure 12A**) and the strength of T cell activation (**Figure 12B**) shown for each clone in the FAP-targeted trivalent format. Interestingly, a negative correlation was observed between the strength of binding strength to OX40 and agonism of OX40 signalling. The ability to dynamically recruit signaling OX40 receptor units might be stronger in a low affinity format with high avidity. The low affinity

might guarantee turn-over of receptor units by quick catch and release, whereas the avidity still guarantees a clustering of several OX40 molecules at once, and hypercrosslinking by FAP for optimal receptor signaling.

Example 6

5 Generation of bispecific antibodies targeting 4-1BB and fibroblast activation protein (FAP)

6.1 Generation of bispecific, trivalent antibodies targeting 4-1BB and fibroblast activation protein (FAP)

Bispecific agonistic 4-1BB constructs with trivalent binding for 4-1BB and trivalent binding for FAP were prepared.

- 10 To enhance binding of Fab molecules to 4-1BB, and for enhanced cross-linking of these receptors, trimerized Fab molecules targeting OX40 were generated by the method described in WO 2014/180754 A1.

- Fab genes of the 4-1BB binders (VHCH1) were connected by a (Gly₄Ser)₂ linker to a short trimerisation domain derived from human cartilage matrix protein (CMP) (Uniprot
15 Accession: P21941; Residues 454 to 496, SEQ ID NO:1) by standard recombinant DNA technologies. The cysteine residues forming interchain disulfide bridges at positions 458 and 460 were used together with the coiled coil domain comprising residues 467 to 495. Downstream of the CMP domain, a (Gly₄Ser)₄ linker was fused to connect the anti-FAP binding moiety to the trimerisation domain. The FAP binding moieties were comprised of disulfide stabilized scFv
20 fragments (H44/L100) of the FAP specific binder (28H1).

The CMP chain and the anti-4-1BB light chain were co-expressed, resulting in the production of bispecific, trimeric molecules as depicted in **Figure 2B**. The base pair and amino acid sequences for the constructs are shown in **Table 25** and **Table 26**, respectively.

Table 25. Base pair sequences of mature bispecific trivalent anti-4-1BB, anti-FAP human

Clone (4-1BB clone/FA P clone)		SEQ ID NO.	Base pair sequences

3+3 12B3/28 H1	VHCH1(4-1BB)- CMPtd-scFv (28H1)	238	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGA AGCCTGGGTCTCTCGGTGAAGGTCTCCTGCAAGGCCTCC GGAGGCACATTCAGCAGCTACGCTATAAGCTGGGTGC GACAGGCCCTGGACAAGGGCTGGAGTGGATGGGAGG GATCATCCCTATCTTTGGTACAGCAAACCTACGCACAGA AGTTCCAGGGCAGGGTCACCATTACTGCAGACAAATC CACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGA TCTGAGGACACCGCCGTGTATTACTGTGCGAGATCTGA ATTCCGTTTCTACGCTGACTTCGACTACTGGGGCCAAG GGACCACCGTGACCGTCTCGAGTGCTAGCACAAAGGG ACCTAGCGTGTTCCCCCTGGCCCCCAGCAGCAAGTCTA CATCTGGCGGAACAGCCGCCCTGGGCTGCCTCGTGAA GGACTACTTTCCCGAGCCCGTGACCGTGTCTGGAAGT CTGGCGCTCTGACAAGCGGCGTGACACCTTTCCAGCC GTGCTGCAGAGCAGCGGCCTGTACTCTCTGAGCAGCG TCGTGACAGTGCCAGCAGCTCTCTGGGCACCCAGAC CTACATCTGCAACGTGAACCACAAGCCCAGCAACACC AAGGTGGACAAGAAGGTGGAACCCAAGAGCTGCGAC GGCGGAGGGGGATCTGGCGGCGGAGGATCCGAGGAA GATCCTTGCGCCTGCGAGAGCCTCGTGAAGTTCCAGGC CAAGGTGGAAGGACTGCTGCAGGCCCTGACCCGGAAA CTGGAAGCCGTGTCCAAGCGGCTGGCCATCCTGGAAA ACACCGTGGTGTCCGGAGGCGGGGGTAGCGGCGGAGG GGGCTCTGGCGGTGGCGGGTCTGGAGGCGGGGGTTCA GAAGTGCAGCTGCTGGAATCTGGCGGCGGACTGGTGC AGCCTGGCGGATCTCTGAGACTGAGCTGTGCCGCCAG CGGCTTCACCTTTAGCAGCCACGCCATGAGCTGGGTGC GCCAGGCCCTGGAAAGTGCCTGGAATGGGTGTCCGC CATCTGGGCCAGCGGCGAGCAGTACTACGCCGATAGC GTGAAGGGCCGGTTACCATCAGCCGGGACAACAGCA AGAACACCCTGTACCTGCAGATGAACAGCCTGCGGGC CGAGGACACCGCCGTGTACTATTGTGCCAAGGGCTGG CTGGGCAACTTCGACTATTGGGGCCAGGGCACCCCTCGT GACCGTGTCTAGCGGAGGGGGCGGAAGTGGTGGCGGG GGAAGCGGCGGGGGTGGCAGCGGAGGGGGCGGATCT GAAATTGTGCTGACCCAGAGCCCTGGCACCCCTGAGCC TGTCTCCAGGCGAAAGAGCCACACTGAGCTGCAGAGC CAGCCAGAGCGTGTCCAGAAGCTACCTGGCCTGGTAT CAGCAGAAGCCCGGACAGGCCCCCAGACTGCTGATCA TCGGCGCCTCTACAAGAGCCACCGGCATCCCCGATAG ATTCAGCGGCTCTGGCAGCGGCACCGACTTCACCCTGA CCATCAGCAGACTGGAACCCGAGGACTTTGCCGTGTA TACTGCCAGCAGGGCCAAGTGATCCCCCCCACCTTTG GCTGTGGCACAAAGGTGGAAATCAAA
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	VLCL- Light chain 1 (4-1BB)	239	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGC ATCTGTAGGAGACCGTGTCAACCATCACTTGCCGTGCCA GTCAGAGTATTAGTAGCTGGTTGGCCTGGTATCAGCAG AAACCAGGGAAAGCCCCCTAAGCTCCTGATCTATGATG CCTCCAGTTTGGAAAGTGGGGTCCCATCACGTTTCAGC GGCAGTGGATCCGGGACAGAATTCACTCTCACCATCA GCAGCTTGCAGCCTGATGATTTTGCAACTTATTACTGC CAACAGTATCATTCGTATCCGCAGACGTTTGGCCAGGG CACCAAAGTCGAGATCAAGCGTACGGTGGCTGCACCA TCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAA ATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACT TCTATCCCAGAGAGGGCCAAAGTACAGTGGAAGGTGGA TAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTC ACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCA GCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAA ACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGC CTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAG AGTGT
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3+3 25G7/28 H1	VHCH1(4-1BB)- CMPtd-scFv (28H1)	240	GAGGTGCAATTGTTGGAGTCTGGGGGAGGCTTGGTAC AGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCC GGATTCACCTTTAGCAGTTATGCCATGAGCTGGGTCCG CCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCT ATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACT CCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTC CAAGAACACGCTGTATCTGCAGATGAACAGCCTGAGA GCCGAGGACACGGCCGTATATTACTGTGCGCGTGACG ACCCGTGGCCGCCGTTCGACTACTGGGGCCAAGGAAC CCTGGTCACCGTCTCGAGTGCTAGCACAAAGGGACCT AGCGTGTTCCCCCTGGCCCCCAGCAGCAAGTCTACATC TGGCGGAACAGCCGCCCTGGGCTGCCTCGTGAAGGAC TACTTTCCCGAGCCCGTGACCGTGTCTGGAAGTCTGG CGCTCTGACAAGCGGCGTGACACCTTTCCAGCCGTGC TGCAGAGCAGCGGCCTGTACTCTCTGAGCAGCGTCGT GACAGTGCCCAGCAGCTCTCTGGGCACCCAGACCTAC ATCTGCAACGTGAACCACAAGCCCAGCAACACCAAGG TGGACAAGAAGGTGGAACCCAAGAGCTGCGACGGCG GAGGGGGATCTGGCGGCGGAGGATCCGAGGAAGATCC TTGCGCCTGCGAGAGCCTCGTGAAGTTCAGGCCAAG GTGGAAGGACTGCTGCAGGCCCTGACCCGGAAACTGG AAGCCGTGTCCAAGCGGCTGGCCATCCTGGAAAACAC CGTGGTGTCCGGAGGCGGGGGTAGCGGCGGAGGGGGC TCTGGCGGTGGCGGGTCTGGAGGCGGGGGTTTCAAG TGCAGCTGCTGGAATCTGGCGGCGGACTGGTGCAGCC TGGCGGATCTCTGAGACTGAGCTGTGCCGCCAGCGGC TTCACCTTTAGCAGCCACGCCATGAGCTGGGTGCGCCA GGCCCCCTGGAAAGTGCCTGGAATGGGTGTCCGCCATC TGGGCCAGCGGCGAGCAGTACTACGCCGATAGCGTGA AGGGCCGGTTCACCATCAGCCGGGACAACAGCAAGAA CACCTGTACCTGCAGATGAACAGCCTGCGGGCCGAG GACACCGCCGTGTACTATTGTGCCAAGGGCTGGCTGG GCAACTTCGACTATTGGGGCCAGGGCACCTCGTGAC CGTGTCTAGCGGAGGGGGCGGAAGTGGTGGCGGGGGA AGCGGCGGGGGTGGCAGCGGAGGGGGCGGATCTGAA ATTGTGCTGACCCAGAGCCCTGGCACCTGAGCCTGTC TCCAGGCGAAAGAGCCACACTGAGCTGCAGAGCCAGC CAGAGCGTGTCCAGAAGCTACCTGGCCTGGTATCAGC AGAAGCCCCGACAGGCCCCCAGACTGCTGATCATCGG CGCCTCTACAAGAGCCACCGGCATCCCCGATAGATTC AGCGGCTCTGGCAGCGGCACCGACTTCACCTGACCA TCAGCAGACTGGAACCCGAGGACTTTGCCGTGTATTAC TGCCAGCAGGGCCAAGTGATCCCCCCCACCTTTGGCTG TGGCACAAAGGTGGAAATCAAA
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	VLCL- Light chain 1 (4-1BB)	241	TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGC CTTGGGACAGACAGTCAGGATCACATGCCAAGGAGAC AGCCTCAGAAGTTATTATGCAAGCTGGTACCAGCAGA AGCCAGGACAGGCCCTGTACTTGTTCATCTATGGTAAA AACAACCGGCCCTCAGGGATCCCAGACCGATTCTCTG GCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACT GGGGCTCAGGCGGAAGATGAGGCTGACTATTACTGTA ACTCCCTTGATAGGCGCGGTATGTGGGTATTCGGCGGA GGGACCAAGCTGACCGTCCTAGGTCAACCCAAGGCTG CCCCCAGCGTGACCCTGTTCCCCCCCAGCAGCGAGGA ACTGCAGGCCAACAAGGCCACCCTGGTCTGCCTGATC AGCGACTTCTACCCAGGCGCCGTGACCGTGGCCTGGA AGGCCGACAGCAGCCCCGTGAAGGCCGGCGTGGAGAC CACCACCCCCAGCAAGCAGAGCAACAACAAGTACGCC GCCAGCAGCTACCTGAGCCTGACCCCCGAGCAGTGGA AGAGCCACAGGTCCTACAGCTGCCAGGTGACCCACGA GGGCAGCACCGTGGAGAAAACCGTGGCCCCCACCAGAG TGCAGC
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3+3 11D5/28 H1	VHCH1(4-1BB)- CMPtd-scFv (28H1)	242	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGA AGCCTGGGTCTCTCGGTGAAGGTCTCCTGCAAGGCCTCC GGAGGCACATTCAGCAGCTACGCTATAAGCTGGGTGC GACAGGCCCTGGACAAGGGCTCGAGTGGATGGGAGG GATCATCCCTATCTTTGGTACAGCAAACCTACGCACAGA AGTTCCAGGGCAGGGTCACCATTACTGCAGACAAATC CACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGA TCTGAGGACACCGCCGTGTATTACTGTGCGAGATCTAC TCTGATCTACGGTTACTTCGACTACTGGGGCCAAGGGA CCACCGTGACCGTCTCCTCAGCTAGCACAAAGGGACC TAGCGTGTTCCCCCTGGCCCCCAGCAGCAAGTCTACAT CTGGCGGAACAGCCGCCCTGGGCTGCCTCGTGAAGGA CTACTTTCCCGAGCCCGTGACCGTGTCTGGAACCTCTG GCGCTCTGACAAGCGGCGTGCACACCTTTCCAGCCGTG CTGCAGAGCAGCGGCCTGTACTCTCTGAGCAGCGTCGT GACAGTGCCCAGCAGCTCTCTGGGCACCCAGACCTAC ATCTGCAACGTGAACCACAAGCCCAGCAACACCAAGG TGGACAAGAAGGTGGAACCCAAGAGCTGCGACGGCG GAGGGGGATCTGGCGGCGGAGGATCCGAGGAAGATCC TTGCGCCTGCGAGAGCCTCGTGAAGTTCCAGGCCAAG GTGGAAGGACTGCTGCAGGCCCTGACCCGGAAACTGG AAGCCGTGTCCAAGCGGCTGGCCATCCTGGAAAACAC CGTGGTGTCCGGAGGCGGGGGTAGCGGCGGAGGGGGC TCTGGCGGTGGCGGGTCTGGAGGCGGGGGTTTCAAG TGCAGCTGCTGGAATCTGGCGGCGGACTGGTGCAGCC TGGCGGATCTCTGAGACTGAGCTGTGCCGCCAGCGGC TTCACCTTTAGCAGCCACGCCATGAGCTGGGTGCGCCA GGCCCCCTGGAAAGTGCCTGGAATGGGTGTCCGCCATC TGGGCCAGCGGCGAGCAGTACTACGCCGATAGCGTGA AGGGCCGGTTCACCATCAGCCGGGACAACAGCAAGAA CACCTGTACCTGCAGATGAACAGCCTGCGGGCCGAG GACACCGCCGTGTACTATTGTGCCAAGGGCTGGCTGG GCAACTTCGACTATTGGGGCCAGGGCACCCCTCGTGAC CGTGTCTAGCGGAGGGGGCGGAAGTGGTGGCGGGGGA AGCGGCGGGGGTGGCAGCGGAGGGGGCGGATCTGAA ATTGTGCTGACCCAGAGCCCTGGCACCCCTGAGCCTGTC TCCAGGCGAAAGAGCCACACTGAGCTGCAGAGCCAGC CAGAGCGTGTCCAGAAGCTACCTGGCCTGGTATCAGC AGAAGCCCCGACAGGCCCCCCAGACTGCTGATCATCGG CGCCTCTACAAGAGCCACCGGCATCCCCGATAGATTC AGCGGCTCTGGCAGCGGCACCGACTTCACCTGACCA TCAGCAGACTGGAACCCGAGGACTTTGCCGTGTATTAC TGCCAGCAGGGCCAAGTGATCCCCCCCACCTTTGGCTG TGGCACAAAGGTGGAAATCAAA
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	VLCL- Light chain 1 (4-1BB)	243	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGC ATCTGTAGGAGACCGTGTCAACCATCACTTGCCGTGCCA GTCAGAGTATTAGTAGCTGGTTGGCCTGGTATCAGCAG AAACCAGGGAAAGCCCCCTAAGCTCCTGATCTATGATG CCTCCAGTTTGGAAAGTGGGGTCCCATCACGTTTCAGC GGCAGTGGATCCGGGACAGAATTCACTCTCACCATCA GCAGCTTGCAGCCTGATGATTTTGCAACTTATTACTGC CAACAGCTTAATTCGTATCCTCAGACGTTTGGCCAGGG CACCAAAGTCGAGATCAAGCGTACGGTGGCTGCACCA TCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAA ATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACT TCTATCCCAGAGAGGGCCAAAGTACAGTGGAAGGTGGA TAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTC ACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCA GCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAA ACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGC CTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAG AGTGT
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3+3 9B11/28 H1	VHCH1(4-1BB)- CMPtd-scFv (28H1)	244	CAGGTGCAATTGGTGCAGTCTGGGGCTGAGGTGAAGA AGCCTGGGTCTCTCGGTGAAGGTCTCCTGCAAGGCCTCC GGAGGCACATTCAGCAGCTACGCTATAAGCTGGGTGC GACAGGCCCTGGACAAGGGCTCGAGTGGATGGGAGG GATCATCCCTATCTTTGGTACAGCAAACCTACGCACAGA AGTTCCAGGGCAGGGTCACCATTACTGCAGACAAATC CACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGA TCTGAGGACACCGCCGTGTATTACTGTGCGAGATCTTC TGGTGCTTACCCGGGTTACTTCGACTACTGGGGCCAAG GGACCACCGTGACCGTCTCCTCAGCTAGCACAAAGGG ACCTAGCGTGTTCCCCCTGGCCCCCAGCAGCAAGTCTA CATCTGGCGGAACAGCCGCCCTGGGCTGCCTCGTGAA GGACTACTTTCCCGAGCCCGTGACCGTGTCTGGAACCT CTGGCGCTCTGACAAGCGGCGTGCACACCTTTCCAGCC GTGCTGCAGAGCAGCGGCCTGTACTCTCTGAGCAGCG TCGTGACAGTGCCAGCAGCTCTCTGGGCACCCAGAC CTACATCTGCAACGTGAACCACAAGCCCAGCAACACC AAGGTGGACAAGAAGGTGGAACCCAAGAGCTGCGAC GGCGGAGGGGGATCTGGCGGCGGAGGATCCGAGGAA GATCCTTGCGCCTGCGAGAGCCTCGTGAAGTTCCAGGC CAAGGTGGAAGGACTGCTGCAGGCCCTGACCCGGAAA CTGGAAGCCGTGTCCAAGCGGCTGGCCATCCTGGAAA ACACCGTGGTGTCCGGAGGCGGGGGTAGCGGCGGAGG GGGCTCTGGCGGTGGCGGGTCTGGAGGCGGGGGTTCA GAAGTGCAGCTGCTGGAATCTGGCGGCGGACTGGTGC AGCCTGGCGGATCTCTGAGACTGAGCTGTGCCGCCAG CGGCTTCACCTTTAGCAGCCACGCCATGAGCTGGGTGC GCCAGGCCCTGGAAAGTGCCTGGAATGGGTGTCCGC CATCTGGGCCAGCGGCGAGCAGTACTACGCCGATAGC GTGAAGGGCCGGTTACCATCAGCCGGGACAACAGCA AGAACACCCTGTACCTGCAGATGAACAGCCTGCGGGC CGAGGACACCGCCGTGTACTATTGTGCCAAGGGCTGG CTGGGCAACTTCGACTATTGGGGCCAGGGCACCCCTCGT GACCGTGTCTAGCGGAGGGGGCGGAAGTGGTGGCGGG GGAAGCGGCGGGGGTGGCAGCGGAGGGGGCGGATCT GAAATTGTGCTGACCCAGAGCCCTGGCACCCCTGAGCC TGTCTCCAGGCGAAAGAGCCACACTGAGCTGCAGAGC CAGCCAGAGCGTGTCCAGAAGCTACCTGGCCTGGTAT CAGCAGAAGCCCGGACAGGCCCCCAGACTGCTGATCA TCGGCGCCTCTACAAGAGCCACCGGCATCCCCGATAG ATTCAGCGGCTCTGGCAGCGGCACCGACTTCACCCTGA CCATCAGCAGACTGGAACCCGAGGACTTTGCCGTGTA TACTGCCAGCAGGGCCAAGTGATCCCCCCCACCTTTG GCTGTGGCACAAAGGTGGAAATCAAA
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	VLCL-Light chain 1 (4-1BB)	245	GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGC ATCTGTAGGAGACCGTGTACCATCACTTGCCGTGCCA GTCAGAGTATTAGTAGCTGGTTGGCCTGGTATCAGCAG AAACCAGGGAAAGCCCCCTAAGCTCCTGATCTATGATG CCTCCAGTTTGGAAAGTGGGGTCCCATCACGTTTCAGC GGCAGTGGATCCGGGACAGAATTCCTCTCACCATCA GCAGCTTGCAGCCTGATGATTTTGCAACTTATTACTGC CAACAGGTTAATTCTTATCCGCAGACGTTTGGCCAGGG CACCAAAGTCGAGATCAAGCGTACGGTGGCTGCACCA TCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAA ATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACT TCTATCCCAGAGAGGGCCAAAGTACAGTGGAAAGGTGGA TAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTC ACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCA GCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAA ACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGC CTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAG AGTGT
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Table 26. Amino acid sequences of the mature, bispecific trivalent anti-4-1BB, anti-FAP human

Clone		SEQ ID NO.	Amino acid sequences
12B3	VHCH1(4-1BB)-CMPtd-scFv (28H1)	246	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQ APGQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTA YMELSSLRSEDNAVYYCARSEFRFYADFDYWGGQTTVT SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT SWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQT YICNVNHKPSNTKVDKKVEPKSCDGGGSGGGGSEEDPC ACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVVS GGGSGGGGSGGGGSGGGGSEVQLLESGLLVQPGGSLRL SCAASGFTFSSHAMSWVRQAPGKCLEWVSAIWASGEQYY ADSVKGRFTISRDNSTNTLYLQMNSLRAEDTAVYYCAKG WLGNFQDYWGQGTLLTVSSGGGSGGGGSGGGGSGGGGS EIVLTQSPGTLSLSPGERATLSCRASQSVSRSYLAWYQQK PQQAPRLLIIGASTRATGIPDRFSGSGSGTDFTLTISRLE PEDFNAVYYCQQGQVIPPTFGCGTKVEIK
	VLCL-Light chain 1 (4-1BB)	247	DIQMTQSPSTLSASVGDRTITCRASQSISSWLAWYQQKPG KAPKLLIYDASSLESGVPSRFSGSGSGTEFTLTISLQPD DFATYYCQQYHSYPQTFGQGTKEIKRTVAAPSVFIFPPS DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSYSLSSLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC

25G7	VHCH1(4-1BB)-CMPtd-scFv (28H1)	248	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLY LQMNSLRAEDTAVYYCARDDPWPPFDYWGQGT LVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDGGGSGGGGSEEDPCAC ESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVVSGGG GSGGGGSGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSC AASGFTFSSHAMSWVRQAPGKCLEWVSAIWASGEQYYAD SVKGRFTISRDN SKNTLY LQMNSLRAEDTAVYYCAKGWL GNFDYWGQGT LVTVSSGGGGSGGGGSGGGGSGGGGSEIV LTQSPGTLSLSPGERATLSCRASQSVSRSYLAWYQQKPGQ APRLLIIGASTRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV YYCQQGQVIPPTFGCGTKVEIK
	VLCL-Light chain 1 (4-1BB)	249	SSELTQDPAVSVALGQTVRITCQGDLSRSYYASWYQQKPG QAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAED EADYYCNSLDRGMWVFGGGTKLTVRTVAAPSVFIFPPSD EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE SVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC
11D5	VHCH1(4-1BB)-CMPtd-scFv (28H1)	250	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSTLIYGYFDYWGQGT TTVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDGGGSGGGGSEEDPCAC ESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVVSGGG GSGGGGSGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSC AASGFTFSSHAMSWVRQAPGKCLEWVSAIWASGEQYYAD SVKGRFTISRDN SKNTLY LQMNSLRAEDTAVYYCAKGWL GNFDYWGQGT LVTVSSGGGGSGGGGSGGGGSGGGGSEIV LTQSPGTLSLSPGERATLSCRASQSVSRSYLAWYQQKPGQ APRLLIIGASTRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV YYCQQGQVIPPTFGCGTKVEIK
	VLCL-Light chain 1 (4-1BB)	251	DIQMTQSPSTLSASVGDRVITITCRASQSISSWLAWYQQKPG KAPKLLIYDASSLESGVPSRFSGSGSGTEFTLTISLQPDDE ATYYCQQLNSYPQTFGQGTKVEIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESV TEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSS PVTKSFNREGC

9B11	VHCH1(4-1BB)-CMPtd-scFv (28H1)	252	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQ APGQGLEWMGGIPIFGTANYAQKFQGRVTITADKSTSTA YMELSSLRSEDVAVYYCARSSGAYPGYFDYWGGQTTVT SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT YICNVNHKPSNTKVDKKVEPKSCDGGGSGGGGSEEDPC ACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVVSG GGGSGGGGSGGGGSGGGGSEVQLLES GGGLVQPGGSLRL SCAASGFTFSSHAMSWVRQAPGKCLEWVSAIWASGEQYY ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKG WLG NFDYWGGQTLTVSSGGGSGGGGSGGGGSGGGGSG EIVLTQSPGTLSLSPGERATLSCRASQSVSRSYLAWYQQK PQQAPRLLIIGASTRATGIPDRFSGSGSGTDFTLTISRLE PEDFVAVYYCQQGQVIPPTFGCGTKVEIK
	VLCL-Light chain 1 (4-1BB)	253	DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPG KAPKLLIYDASSLESGVPSRFSGSGSGTEFTLTISSLQPD DFATYYCQQVNSYPQTFGGQTKVEIKRTVAAPSVFIFPPS DEQLKSGTASVVCLLNFPYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC

In addition, an “untargeted” 25G7 construct was prepared, in which the VH and VL of the anti-FAP binder were replaced by a germline control, termed DP47, which does not display binding to FAP.

- 5 The VHCH1 (4-1BB) – CMPtd – scFc (FAP) sequence (and the corresponding VLCL sequence of the anti-4-1BB Fab) are operatively fused to a recombinant chimeric MPSV promoter for expression in mammalian cells. The used expression vectors also contain the oriP sequence for stable maintenance of plasmids in cells providing the Epstein Barr large nuclear antigen (EBNA). In addition a synthetic polyA signal sequence is located at the 3' end of the
- 10 CDS.

- For production of the trimeric, bispecific anti-4-1BB, anti-FAP molecules constructs were produced by co-transfecting HEK293-EBNA cells with the mammalian expression vectors using polyethylenimine. The cells were transfected with the corresponding expression vectors in a 1:1 ratio (“vector CH-VH CMPtd disulphide-stabilised scFv” : “vector CL-VL”). Transfection
- 15 was performed as described for the anti-4-1BB, anti-FAP constructs in **Example 3.1** hereinabove.

The secreted protein was purified from cell culture supernatants first by affinity chromatography via the CH1 domain of human IgG antibodies, followed by size exclusion chromatography. For affinity chromatography, the supernatant was loaded on a column packed with CaptureSelect IgG-CH1 matrix (Column Volume = 1 ml; BAC, The Netherlands) and

equilibrated with 5 ml 50 mM Tris(hydroxymethyl)-aminomethan (TRIS), 100 mM Glycine, 150 mM sodium chloride, pH 8.0. Unbound protein was removed by washing with at least ten column volumes 50 mM TRIS, 100 mM Glycine, 150 mM sodium chloride, pH 8.0. The bound protein was eluted in a linear pH-gradient over 20 column volumes from 50 mM TRIS, 100 mM Glycine, 150 mM sodium chloride pH 8.0 to pH 2.0. The column was subsequently washed with 10 column volume 50 mM TRIS, 100 mM Glycine, 150 mM sodium chloride, pH 2.0.

The protein solution (i.e. the collected fraction) was neutralized by addition of 1/40 (v/v) of 2M Tris, pH 8.0, and was followed by a concentration step. Finally, the protein was filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 20 mM Histidine, 140 mM sodium chloride, 0.01% (v/v) Tween-20 solution of pH 6.0.

Analysis confirmed a homogeneous preparation of the trimerized molecules (**Table 27**).

Table 27. Biochemical analysis of huCMP containing bispecific 4-1BB/FAP constructs.

Construct	SEC Trimer [%]
12B3 huCMP FAP	98.1 %
25G7 huCMP FAP	97.7 %
9B11 huCMP FAP	96.3 %

In addition, an control molecule DP47 x FAP 3+3 was prepared, in which the VH and VL domains of the 4-1BB binding region were replaced by the germline control DP47, which does not display binding to 4-1BB.

Example 7

Binding of trimeric, bispecific antigen binding molecules targeting 4-1BB and FAP

7.1 Analysis of simultaneous binding to 4-1BB and FAP by surface plasmon resonance analysis

The ability of the trimeric, bispecific antibodies to bind simultaneously to human 4-1BB Fc(kih) and human FAP was assessed by surface plasmon resonance (SPR) analysis. All SPR experiments were performed on a Biacore T200 at 25 °C with HBS-EP as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, Biacore, Freiburg/Germany).

Biotinylated human 4-1BB Fc(kih) was directly coupled to the chip surface of a streptavidin (SA) sensor chip. Immobilization levels up to 400 resonance units (RU) were used.

The bispecific antibodies targeting 4-1BB and FAP were over the chip surface at a concentration of 200 nM, and at a flow of 30 μ L/minute for 90 seconds, with a dissociation phase set to zero sec. Human FAP was injected as the second analyte, at a concentration of 500 nM, and at a flow rate of 30 μ L/minute for 90 seconds (**Figure 3B**). Dissociation was monitored for 120 sec. Bulk refractive index differences were corrected for by subtracting the response obtained in a reference flow cell, where no protein was immobilized.

All trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules were found to be able to bind simultaneously to human 4-1BB and human FAP (**Figure 13A to 13C**).

7.2 Binding to human 4-1BB expressing cells: analysis of binding to resting (naïve) and activated human peripheral mononuclear blood mononuclear cells (PBMCs)

Human PBMCs were isolated from buffy coat of healthy donors obtained from the blood donation center Zurich (Blutspendezentrum Schlieren, Switzerland) by Ficoll density gradient centrifugation. 50 mL Falcon-tubes were supplied with 15 mL Histopaque 1077 (SIGMA life science, Cat.-No. 10771). The buffy coat was diluted 1:1 with sterile DPBS (GIBCO life technologies, Cat.-No. 14190-136) and the Histopaque 1077 was overlaid with this cell suspension. Tubes were centrifuged 30 min 450 x g at room temperature, with low acceleration and no break. PBMCs were harvested from the interphase and washed several times with DPBS.

The isolated PBMCs were used in experiments either directly after isolation (for analysis of binding to resting (naïve) human PBMCs), or following stimulation to provide for high expression human 4-1BB on the cell surface of T cells. For stimulation and induction of 4-1BB expression, PBMCs were resuspended to 1×10^6 cells/mL in RPMI 1640 (GIBCO life technologies, Cat.-No. 42401-042) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO life science, Cat.-No. 16000-044, Lot 941273, gamma irradiated mycoplasma free, heat inactivated 35 min 56 °C), 2 mM L-alanyl-L-glutamine (GlutaMAX-I, GIBCO life technologie, Cat.-No. 35050-038), 1% (v/v) MEM-Non essential Aminoacid Solution (100x, SIGMA life science, Cat.-No. M7145), 1 mM Sodium-Pyruvate (100 mM stock, SIGMA-Aldrich Cat.-No. S8636), 50 μ M beta-Mercaptoethanol, 2 μ g/mL PHA-L (SIGMA life science, Cat.-No. L2769) and 200 U/mL Proleukin (Novartis Pharma Schweiz AG, CHCLB-P-476-700-10340, Lot AA4493BAL). Cells were seeded to 6-well tissue culture plates and incubated for 4 days at 37°C in a 5% CO₂ atmosphere. After 4 days cells were harvested, counted and resuspended in RPMI 1640 supplied with 10% (v/v) FBS, 2 mM L-alanyl-L-glutamine, 1% (v/v) MEM-non-essential aminoacid solution 1 mM Sodium-Pyruvate, 50 μ M beta-Mercaptoethanol, and 200 U/mL Proleukin to 10^6 cells/mL. Cells were seeded to 6-well tissue culture plates, which had been pre-

coated overnight with DPBS containing 10 µg/mL LEAF purified anti-human CD3 mouse IgG2a (clone OKT3, BioLegend, Cat.-No. 317315) and 2 µg/mL functional grade purified anti-human CD28 mouse IgG1 (clone 28.2, BioLegend, Cat.-No. 16-0289-85). Cells were reactivated for 24 hours at 37°C and 5% CO₂.

- 5 For the assays, naïve or activated human PBMCs were resuspended to 1×10^6 cells/mL in 4 °C DPBS, and 100 µL were seeded to wells of 96-well round bottomed suspension cell plates.

- Plates were centrifuged, and supernatants were removed. If dead cells were not stained by using DAPI cells were resuspended in 100 µL/well DPBS containing 1:1000 diluted LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technology Molecular Probes, Cat.-No. L34957),
10 incubated for 30 min at 4°C and washed once with DPBS.

- Cells were subsequently resuspended in 50 µL/well FACS buffer (DPBS containing 2% (v/v) FBS, 50 mM EDTA, 7.5 mM Sodium-Azide) containing different concentrations of 4-1BB-binding human IgG1 P329G LALA, or trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules and incubated for 2 h at 4 °C. Cells were then washed three times with DPBS and
15 resuspended in 50 µL/well FACS buffer containing 2.5 µg/mL R-Phycoerythrin AffiniPure Anti-Human IgG F(ab')₂ fragment specific goat F(ab')₂ fragment (Jackson immunoresearch, Cat.-No. 109-116-097) and incubated for 30 min at 4°C. If dead cells were not stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, cells were resuspended in 100 µL/well FACS buffer containing 0.1 µg/mL DAPI (Santa Cruz Biotech, Cat.-No. Sc-3598). If dead cells were stained
20 with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, they were fixed for 10 min in 1% PFA and then resuspended in 100 µL FACS buffer. Cells were acquired using three-laser CantoII or five-laser LSRFortessa (BD) and analyzed using DIVA-software.

Table 28. EC₅₀ values for binding to human 4-1BB expressed on human activated T cells

construct	EC50 [nM] on CD8	EC50 [nM] on CD4
12B3 huCMP	0.43	0.61
25G7 huCMP	6.61	4.03
12B3 x FAP 3+3	0.82	2.32
25G7 x FAP 3+3	8.90	6.58

- 25 To show that trimerization of anti-4-1BB Fab via huCMP-fusion and targeting to FAP by fusion of trimeric anti-FAP (28H1) scFv did not influence the binding to human 4-1BB, binding to freshly-isolated PBMCs and activated human PBMCs was analysed (**Figure 14**). Because the molecules do not contain an Fc-fragment, an PE-conjugated anti-human IgG F(ab')₂ fragment specific secondary antibody was used for detection. The FAP-targeted scFv- huCMP anti-4-1BB

trimerized clones 25G7 and 12B3 were shown not bind to freshly-isolated human PBMCs, but did bind to 4-1BB-expressing activated human CD8⁺ and CD4⁺ T cells, as expected.

Engineering of the anti-human 4-1BB binding clones 25G7 and 12B3 to the FAP scFv-targeted huCMP trimerized 3+3 format was therefore shown not change their ability to bind to 4-1BB-expressing human T cells. Clone 12B3 was found to bind more strongly to 4-1BB than 25G7.

7.3 Binding to human FAP-expressing and FAP-negative tumor cells

Binding of the trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules to FAP was analysed using NIH/3T3-huFAP clone 39 cell line (described in **Example 5.1**). 2×10^5 of the human FAP-expressing tumor cells were added to wells of round-bottomed suspension cell 96-well plates (Greiner Bio-One, cellstar, Cat.-No. 650185). Cells were washed once with 200 μ L DPBS and resuspended in 100 μ L/well 4 °C DPBS buffer containing 1:5000 diluted Fixable Viability Dye eFluor 450 (eBioscience, Cat. No. 65 0863 18), and the cells then were incubated for 30 minutes at 4°C. Cells were subsequently washed once with 200 μ L 4 °C DPBS buffer, resuspended in 50 μ L/well of 4 °C cold FACS buffer containing different titrated concentrations of 12B3 x FAP 3+3, 25G7 x FAP 3+3, DP47 x FAP 3+3, 25G7 huIgG1 P329G LALA or 12B3 huIgG1 P329G LALA, followed by incubation for 1 hour at 4°C. After washing five times with 200 μ L DPBS/well, cells were stained with 50 μ L/well of 4 °C cold FACS buffer containing 2.5 μ g/mL R-Phycoerythrin AffiniPure Anti-Human IgG F(ab')₂ fragment specific goat F(ab')₂ fragment for 30 minutes at 4 °C. Cells were washed twice with 200 μ L 4 °C FACS buffer, and resuspended in 50 μ L/well DPBS containing 1 % Formaldehyde and stored overnight at 4 °C. The next day cells were resuspended in 100 μ L FACS-buffer and acquired using 5-laser LSR-Fortessa (BD Bioscience with DIVA software).

As shown in **Figure 15** in the FAP-targeted molecules were found to bind efficiently to human FAP-expressing cells, but the DP47-untargeted or parental huIgG1 P293G LALA 25G7 and 12B3 clones did not bind to the human FAP-expressing cells. Therefore, trimeric, bispecific anti-4-1BB, anti-FAP antigen binding molecules are efficiently targeted to FAP-expressing cells.

Example 8

Biological Activity of trimeric, bispecific antigen binding molecules targeting 4-1BB and FAP

8.1 HeLa cells expressing human 4-1BB and reporter gene NF- κ B-luciferase

NF- κ B-luciferase human-4-1BB HeLa clone 26 cells (described in WO 2016156291 A1) were harvested and resuspended in DMEM medium supplied with 10 % (v/v) FBS and 1 % (v/v)

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GlutaMAX-I to a concentration of 0.2×10^6 cells/ml. 100 μ l (2×10^4 cells) of this cell suspension were transferred to wells of a sterile white 96-well flat bottomed tissue culture plate with lid (Greiner Bio-One, Cat. No. 655083), and incubated at 37 °C and 5 % CO₂ overnight. The next day 50 μ L of medium containing titrated 12B3 or 25G7 FAP 3+3 constructs, untargeted huCMP 12B3 or 25G7 constructs, 25G7 or 12B3 huIgG1 P329G LALA parental antibodies, or DP47-untargeted huIgG1 P329G LALA isotype control were added to the wells. FAP-expressing NIH/3T3-huFAP clone 39 cells were subsequently resuspended in DMEM medium supplemented with 10 % (v/v) FBS and 1 % (v/v) GlutaMAX-I to a concentration of 2×10^6 cells/ml, 50 μ L of the cell suspension of FAP-expressing tumor cells was added to each well (1×10^5 cells/well), and plates were incubated for 6 hours at 37 °C and at 5 % CO₂ in an incubator. Cells were washed three times with 200 μ L/well DPBS. 40 μ l freshly prepared Reporter Lysis Buffer (Promega, Cat-No: E3971) were added to each well, and the plates were stored overnight at -20 °C. The next day, the frozen cell plates and Detection Buffer (Luciferase 1000 Assay System, Promega, Cat. No. E4550) were thawed at room temperature. 100 μ L of detection buffer were added to each well, and luciferase activity was measured as soon as possible, using a SpectraMax M5/M5e microplate reader and a SoftMax Pro Software (Molecular Devices).

As shown in **Figure 16B**, FAP-targeted 25G7 or 12B3 3+3 constructs triggered activation of the NF κ B signaling pathway in the reporter cell line in the presence of FAP-expressing tumor cells. By contrast, untargeted huCMP 12B3 or 25G7 constructs, 25G7 or 12B3 huIgG1 P329G LALA parental antibodies and DP47-untargeted huIgG1 P329G LALA isotype control failed to trigger an effect at any of the tested concentrations.

The activation of NF- κ B signalling was found to be strictly dependent on the expression of FAP at the cell surface of tumor cells, as no NF- κ B activation could be detected in the presence of FAP-negative tumor cells (data not shown).

Citations:

- Aggarwal B.B. (2003), Signalling pathways of the TNF superfamily: a double-edged sword. *Nat. Rev. Immunol.* 3(9),745-56.
- Banner D. et al (1993), Crystal structure of the soluble human 55 kd TNF receptor-human TNF
5 beta complex: implications for TNF receptor activation. *Cell* 73, 431-445.
- Beck K., Gambee J., Bohan C., Bächinger H.P. (1996), The C-terminal domain of cartilage matrix protein assembles into a triple-stranded α -helical coiled-coil structure. *J. Mol. Biol.* 256, 909-923.
- Bodmer J., Schneider P. and Tschopp, J. (2002), The molecular architecture of the TNF
10 superfamily. *Trends in Biochemical Sciences* 27(1), 19-26.
- Broll, K., Richter, G., Pauly, S., Hofstaedter, F., and Schwarz, H. (2001). CD137 expression in tumor vessel walls. High correlation with malignant tumors. *Am J Clin Pathol* 115, 543-549.
- Buechele, C., Baessler, T., Schmiedel, B.J., Schumacher, C.E., Grosse-Hovest, L., Rittig, K., and Salih, H.R. (2012). 4-1BB ligand modulates direct and Rituximab-induced NK-cell reactivity in
15 chronic lymphocytic leukemia. *Eur J Immunol* 42, 737-748.
- Choi, B.K., Kim, Y.H., Kwon, P.M., Lee, S.C., Kang, S.W., Kim, M.S., Lee, M.J., and Kwon, B.S. (2009). 4-1BB functions as a survival factor in dendritic cells. *J Immunol* 182, 4107-4115.
- Cuadros, C., Dominguez, A.L., Lollini, P.L., Croft, M., Mittler, R.S., Borgstrom, P., and Lustgarten, J. (2005). Vaccination with dendritic cells pulsed with apoptotic tumors in
20 combination with anti-OX40 and anti-4-1BB monoclonal antibodies induces T cell-mediated protective immunity in Her-2/neu transgenic mice. *Int J Cancer* 116, 934-943.
- Curran, M.A., Kim, M., Montalvo, W., Al-Shamkhani, A., and Allison, J.P. (2011). Combination CTLA-4 blockade and 4-1BB activation enhances tumor rejection by increasing T-cell infiltration, proliferation, and cytokine production. *PLoS One* 6, e19499.
- Diehl, L., van Mierlo, G.J., den Boer, A.T., van der Voort, E., Fransen, M., van Bostelen, L., Krimpenfort, P., Melief, C.J., Mittler, R., Toes, R.E., and Offringa, R. (2002). In vivo triggering through 4-1BB enables Th-independent priming of CTL in the presence of an intact CD28 costimulatory pathway. *J Immunol* 168, 3755-3762.
- Dubrot, J., Milheiro, F., Alfaro, C., Palazon, A., Martinez-Forero, I., Perez-Gracia, J.L., Morales-Kastresana, A., Romero-Trevejo, J.L., Ochoa, M.C., Hervas-Stubbs, S., et al. (2010). Treatment
30 with anti-CD137 mAbs causes intense accumulations of liver T cells without selective antitumor

immunotherapeutic effects in this organ. *Cancer Immunol Immunother* 59, 1223-1233.

Futagawa, T., Akiba, H., Kodama, T., Takeda, K., Hosoda, Y., Yagita, H., and Okumura, K. (2002). Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int Immunol* 14, 275-286.

- 5 Guo, Z., Cheng, D., Xia, Z., Luan, M., Wu, L., Wang, G., and Zhang, S. (2013). Combined TIM-3 blockade and CD137 activation affords the long-term protection in a murine model of ovarian cancer. *J Transl Med* 11, 215.

- Heinisch, I.V., Daigle, I., Knopfli, B., and Simon, H.U. (2000). CD137 activation abrogates granulocyte-macrophage colony-stimulating factor-mediated anti-apoptosis in neutrophils. *Eur J Immunol* 30, 3441-3446.

Ju, S.A., Cheon, S.H., Park, S.M., Tam, N.Q., Kim, Y.M., An, W.G., and Kim, B.S. (2008). Eradication of established renal cell carcinoma by a combination of 5-fluorouracil and anti-4-1BB monoclonal antibody in mice. *Int J Cancer* 122, 2784-2790.

- Kienzle, G., and von Kempis, J. (2000). CD137 (ILA/4-1BB), expressed by primary human monocytes, induces monocyte activation and apoptosis of B lymphocytes. *Int Immunol* 12, 73-82.

Kim, D.H., Chang, W.S., Lee, Y.S., Lee, K.A., Kim, Y.K., Kwon, B.S., and Kang, C.Y. (2008). 4-1BB engagement costimulates NKT cell activation and exacerbates NKT cell ligand-induced airway hyperresponsiveness and inflammation. *J Immunol* 180, 2062-2068.

- Kim, Y.H., Choi, B.K., Oh, H.S., Kang, W.J., Mittler, R.S., and Kwon, B.S. (2009). Mechanisms involved in synergistic anticancer effects of anti-4-1BB and cyclophosphamide therapy. *Mol Cancer Ther* 8, 469-478.

Kwon, B.S., and Weissman, S.M. (1989). cDNA sequences of two inducible T-cell genes. *Proc Natl Acad Sci U S A* 86, 1963-1967.

- Lee, H., Park, H.J., Sohn, H.J., Kim, J.M., and Kim, S.J. (2011). Combinatorial therapy for liver metastatic colon cancer: dendritic cell vaccine and low-dose agonistic anti-4-1BB antibody co-stimulatory signal. *J Surg Res* 169, e43-50.

Li, F., and Ravetch, J.V. (2011). Inhibitory Fcγ receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science* 333, 1030-1034.

- Lin, W., Voskens, C.J., Zhang, X., Schindler, D.G., Wood, A., Burch, E., Wei, Y., Chen, L., Tian, G., Tamada, K., et al. (2008). Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies. *Blood* 112, 699-707.

Melero, I., Johnston, J.V., Shufford, W.W., Mittler, R.S., and Chen, L. (1998). NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell Immunol* 190, 167-172.

5 Melero, I., Shuford, W.W., Newby, S.A., Aruffo, A., Ledbetter, J.A., Hellstrom, K.E., Mittler, R.S., and Chen, L. (1997). Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat Med* 3, 682-685.

Merchant, A.M., Zhu, Z., Yuan, J.Q., Goddard, A., Adams, C.W., Presta, L.G., and Carter, P. (1998). An efficient route to human bispecific IgG. *Nat Biotechnol* 16, 677-681.

10 Morales-Kastresana, A., Sanmamed, M.F., Rodriguez, I., Palazon, A., Martinez-Forero, I., Labiano, S., Hervas-Stubbs, S., Sangro, B., Ochoa, C., Rouzaut, A., et al. (2013). Combined immunostimulatory monoclonal antibodies extend survival in an aggressive transgenic hepatocellular carcinoma mouse model. *Clin Cancer Res* 19, 6151-6162.

15 Murillo, O., Dubrot, J., Palazon, A., Arina, A., Azpilikueta, A., Alfaro, C., Solano, S., Ochoa, M.C., Berasain, C., Gabari, I., et al. (2009). In vivo depletion of DC impairs the anti-tumor effect of agonistic anti-CD137 mAb. *Eur J Immunol* 39, 2424-2436.

Narazaki, H., Zhu, Y., Luo, L., Zhu, G., and Chen, L. (2010). CD137 agonist antibody prevents cancer recurrence: contribution of CD137 on both hematopoietic and nonhematopoietic cells. *Blood* 115, 1941-1948.

20 Nishimoto, H., Lee, S.W., Hong, H., Potter, K.G., Maeda-Yamamoto, M., Kinoshita, T., Kawakami, Y., Mittler, R.S., Kwon, B.S., Ware, C.F., et al. (2005). Costimulation of mast cells by 4-1BB, a member of the tumor necrosis factor receptor superfamily, with the high-affinity IgE receptor. *Blood* 106, 4241-4248.

25 Olofsson, P.S., Soderstrom, L.A., Wagsater, D., Sheikine, Y., Ocaya, P., Lang, F., Rabu, C., Chen, L., Rudling, M., Aukrust, P., et al. (2008). CD137 is expressed in human atherosclerosis and promotes development of plaque inflammation in hypercholesterolemic mice. *Circulation* 117, 1292-1301.

30 Palazon, A., Teixeira, A., Martinez-Forero, I., Hervas-Stubbs, S., Roncal, C., Penuelas, I., Dubrot, J., Morales-Kastresana, A., Perez-Gracia, J.L., Ochoa, M.C., et al. (2011). Agonist anti-CD137 mAb act on tumor endothelial cells to enhance recruitment of activated T lymphocytes. *Cancer Res* 71, 801-811.

Schwarz, H., Valbracht, J., Tuckwell, J., von Kempis, J., and Lotz, M. (1995). ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages. *Blood* 85, 1043-1052.

Shao, Z., and Schwarz, H. (2011). CD137 ligand, a member of the tumor necrosis factor family, regulates immune responses via reverse signal transduction. *J Leukoc Biol* 89, 21-29.

Shi, W., and Siemann, D.W. (2006). Augmented antitumor effects of radiation therapy by 4-1BB antibody (BMS-469492) treatment. *Anticancer Res* 26, 3445-3453.

- 5 Simeone, E., and Ascierto, P.A. (2012). Immunomodulating antibodies in the treatment of metastatic melanoma: the experience with anti-CTLA-4, anti-CD137, and anti-PD1. *J Immunotoxicol* 9, 241-247.

- Snell, L.M., Lin, G.H., McPherson, A.J., Moraes, T.J., and Watts, T.H. (2011). T-cell intrinsic effects of GITR and 4-1BB during viral infection and cancer immunotherapy. *Immunol Rev* 244,
10 197-217.

Stagg, J., Loi, S., Divisekera, U., Ngiow, S.F., Duret, H., Yagita, H., Teng, M.W., and Smyth, M.J. (2011). Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A* 108, 7142-7147.

- Teng, M.W., Sharkey, J., McLaughlin, N.M., Exley, M.A., and Smyth, M.J. (2009). CD1d-based
15 combination therapy eradicates established tumors in mice. *J Immunol* 183, 1911-1920.

von Kempis, J., Schwarz, H., and Lotz, M. (1997). Differentiation-dependent and stimulus-specific expression of ILA, the human 4-1BB-homologue, in cells of mesenchymal origin. *Osteoarthritis Cartilage* 5, 394-406.

- Watts, T.H. (2005). TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol.* 23, 23-68
20

Wei, H., Zhao, L., Li, W., Fan, K., Qian, W., Hou, S., Wang, H., Dai, M., Hellstrom, I., Hellstrom, K.E., and Guo, Y. (2013). Combinatorial PD-1 blockade and CD137 activation has therapeutic efficacy in murine cancer models and synergizes with cisplatin. *PLoS One* 8, e84927.

- Weinberg A.D., Evans D.E., Thalhoffer C, Shi T., and Prell R.A. (2004). The generation of T cell
25 memory: a review describing the molecular and cellular events following OX40 (CD134) engagement. *J. Leukoc. Biol.* 75, 962-972.

Wilcox, R.A., Chapoval, A.I., Gorski, K.S., Otsuji, M., Shin, T., Flies, D.B., Tamada, K., Mittler, R.S., Tsuchiya, H., Pardoll, D.M., and Chen, L. (2002). Cutting edge: Expression of functional CD137 receptor by dendritic cells. *J Immunol* 168, 4262-4267.

- 30 Wilcox, R.A., Tamada, K., Flies, D.B., Zhu, G., Chapoval, A.I., Blazar, B.R., Kast, W.M., and Chen, L. (2004). Ligation of CD137 receptor prevents and reverses established anergy of CD8+

-188-

cytolytic T lymphocytes in vivo. *Blood* 103, 177-184.

Wyzgol A., Müller N., Fick A., Munkel S., Grigoleit G.U., Pfizenmaier K., Wajant H. (2009),
Trimer Stabilization, Oligomerization, and Antibody-mediated cell surface immobilization
improve the activity of soluble trimers of CD27L, CD40L, 41BBL and Glucocorticoid-induced
5 TNF receptor ligand. *J. Immunol.* 183, 1851-1861.

Zhang, X., Voskens, C.J., Sallin, M., Maniar, A., Montes, C.L., Zhang, Y., Lin, W., Li, G.,
Burch, E., Tan, M., et al. (2010). CD137 promotes proliferation and survival of human B cells. *J*
Immunol 184, 787-795.

Claims

1. A trimeric antigen binding molecule comprising three fusion polypeptides, each of the three fusion polypeptides comprising
- 5 (a) a moiety capable of specific binding to a costimulatory TNF receptor family member, (b) a trimerization domain derived from human cartilage matrix protein (huCMP) of amino acid sequence of SEQ ID NO:1, and (c) a moiety capable of specific binding to a target cell antigen.
- 10 2. The trimeric antigen binding molecule of claim 1, wherein the trimerization domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO:2.
3. The trimeric antigen binding molecule of claim 1 or claim 2, wherein the trimerization domain comprises the amino acid sequence of SEQ ID NO:2.
- 15 4. The trimeric antigen binding molecule of any one of claims 1 to 4, wherein the costimulatory TNF receptor family member is selected from OX40 and 4-1BB.
5. The trimeric antigen binding molecule of any one of claims 1 to 4, wherein the moiety
- 20 capable of specific binding to a costimulatory TNF receptor family member binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:3.
6. The trimeric antigen binding molecule of any one of claims 1 to 5, comprising a moiety capable of specific binding to OX40, wherein the moiety comprises a VH comprising
- 25 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5, (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7, and (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ
- 30 ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14, and a VL comprising (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17, (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID
- 35 NO:18, SEQ ID NO:19 and SEQ ID NO:20, and

(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25 and SEQ ID NO:26.

7. The trimeric antigen binding molecule of any one of claims 1 to 6, comprising a moiety
5 capable of specific binding to OX40, wherein the moiety capable of specific binding to OX40
comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%,
99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID
NO:27, SEQ ID NO: 29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37 and
SEQ ID NO:39 and a VL comprising an amino acid sequence that is at least about 95%, 96%,
10 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting
of SEQ ID NO:28, SEQ ID NO: 30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID
NO:38 and SEQ ID NO:40.

8. The trimeric antigen binding molecule of any one of claims 1 to 7, wherein the moiety
15 capable of specific binding to OX40 comprises
(i) a VH comprising the amino acid sequence of SEQ ID NO:27 and a VL comprising the amino
acid sequence of SEQ ID NO:28,
(ii) a VH comprising the amino acid sequence of SEQ ID NO:29 and a VL comprising the amino
acid sequence of SEQ ID NO:30,
20 (iii) a VH comprising the amino acid sequence of SEQ ID NO:31 and a VL comprising the
amino acid sequence of SEQ ID NO:32,
(iv) a VH comprising the amino acid sequence of SEQ ID NO:33 and a VL comprising the
amino acid sequence of SEQ ID NO:34,
(v) a VH comprising the amino acid sequence of SEQ ID NO:35 and a VL comprising the amino
25 acid sequence of SEQ ID NO:36,
(vi) a VH comprising the amino acid sequence of SEQ ID NO:37 and a VL comprising the
amino acid sequence of SEQ ID NO:38, or
(vii) a VH comprising the amino acid sequence of SEQ ID NO:39 and a VL comprising the
amino acid sequence of SEQ ID NO:40.

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9. The trimeric antigen binding molecule of any one of claims 1 to 4, wherein the moiety
capable of specific binding to a costimulatory TNF receptor family member binds to a
polypeptide comprising the amino acid sequence of SEQ ID NO:41.

35 10. The trimeric antigen binding molecule of any one of claims 1 to 4 or 9, comprising a
moiety capable of specific binding to 4-1BB, wherein the moiety comprises a VH comprising
(i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID
NO:42 and SEQ ID NO:43,

- (ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:44 and SEQ ID NO:45, and
- (iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49 and SEQ ID NO:50,
- 5 and a VL comprising
- (iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:51 and SEQ ID NO:52,
- (v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:53 and SEQ ID NO:54, and
- 10 (vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58 and SEQ ID NO:59.

11. The trimeric antigen binding molecule of any one of claims 1 to 4, 9 or 10, comprising a moiety capable of specific binding to 4-1BB, wherein the moiety capable of specific binding to

15 4-1BB comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66 and SEQ ID NO:68 and a VL comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or

20 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67 and SEQ ID NO:69.

12. The trimeric antigen binding molecule of any one of claims 1 to 4 or 9 to 11, wherein the moiety capable of specific binding to 4-1BB comprises

(i) a VH comprising the amino acid sequence of SEQ ID NO:60 and a VL comprising the amino

25 acid sequence of SEQ ID NO:61,

(ii) a VH comprising the amino acid sequence of SEQ ID NO:62 and a VL comprising the amino acid sequence of SEQ ID NO:63,

(iii) a VH comprising the amino acid sequence of SEQ ID NO:64 and a VL comprising the amino acid sequence of SEQ ID NO:65,

30 (iv) a VH comprising the amino acid sequence of SEQ ID NO:66 and a VL comprising the amino acid sequence of SEQ ID NO:67, or

(v) a VH comprising the amino acid sequence of SEQ ID NO:68 and a VL comprising the amino acid sequence of SEQ ID NO:69.

35 13. The trimeric antigen binding molecule of any one of claims 1 to 12, wherein the target cell antigen is selected from the group consisting of Fibroblast Activation Protein (FAP), Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Epidermal Growth Factor Receptor (EGFR), Carcinoembryonic Antigen (CEA), CD19, CD20 and CD33.

14. The trimeric antigen binding molecule of any one of claims 1 to 13, comprising a moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising

- 5 (i) a CDR-H1 comprising the amino acid sequence selected from the group consisting of SEQ ID NO:70 and SEQ ID NO:76,
(ii) a CDR-H2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:71 and SEQ ID NO:77, and
(iii) a CDR-H3 comprising an amino acid sequence selected from the group consisting of SEQ
10 ID NO:72 and SEQ ID NO:78,
and a VL comprising
(iv) a CDR-L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:73 and SEQ ID NO:79,
(v) a CDR-L2 comprising an amino acid sequence selected from the group consisting of SEQ ID
15 NO:74 and SEQ ID NO:80, and
(vi) a CDR-L3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75 and SEQ ID NO:81.

15. The trimeric antigen binding molecule of any one of claims 1 to 14, comprising a
20 moiety capable of specific binding to FAP, wherein the moiety capable of specific binding to FAP comprises a VH comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:82 and SEQ ID NO:84, and a VL comprising an amino acid sequence that is at least
25 about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:83 and SEQ ID NO:85.

16. A polynucleotide encoding the trimeric antigen binding molecule of any one of claims 1 to 15.

- 30 17. An expression vector comprising the polynucleotide of claim 16.

18. A host cell comprising the polynucleotide of claim 16 or the expression vector of claim 17.

- 35 19. A method of producing a trimeric antigen binding molecule, comprising culturing the host cell of claim 18 under conditions suitable for the expression of the trimeric antigen binding molecule, and isolating the trimeric antigen binding molecule.

20. A pharmaceutical composition comprising the trimeric antigen binding molecule of any one of claims 1 to 15 and at least one pharmaceutically acceptable excipient

21. The trimeric antigen binding molecule of claims 1 to 15 for use as a medicament.

5

22. The trimeric antigen binding molecule of claims 1 to 15 for use

(i) in stimulating T cell response,

(ii) in supporting survival of activated T cells,

(iii) in the treatment of infections,

10 (iv) in the treatment of cancer,

(v) in delaying progression of cancer, or

(vi) in prolonging the survival of a patient suffering from cancer.

23. The trimeric antigen binding molecule of any one of claims 1 to 15, or the
15 pharmaceutical composition of claim 20 for use in the treatment of cancer.

24. Use of the trimeric antigen binding molecule of any one of claims 1 to 15, or the
pharmaceutical composition of claim 20, in the manufacture of a medicament for the treatment
of cancer.

20

25. A method of treating an individual having cancer comprising administering to the
individual an effective amount of the trimeric antigen binding molecule of any one of claims 1 to
15, or the pharmaceutical composition of claim 20.

25

Fig. 1

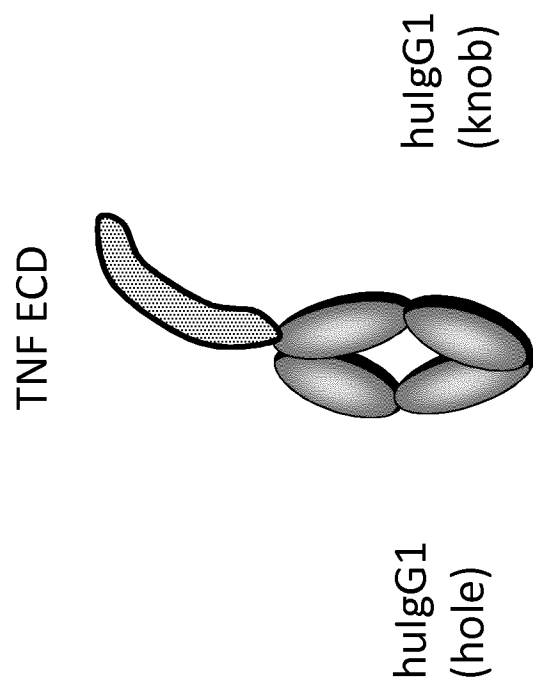
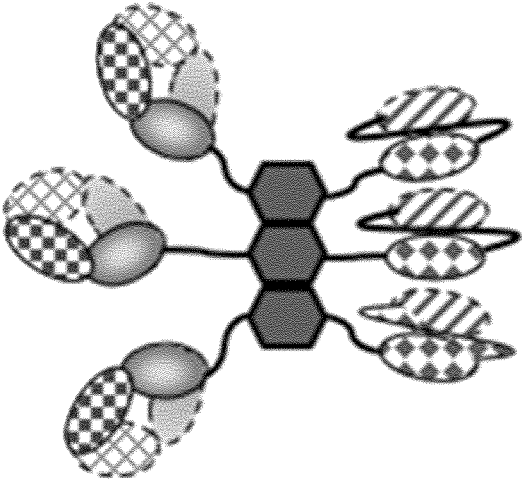


Fig. 2

FIG.2A

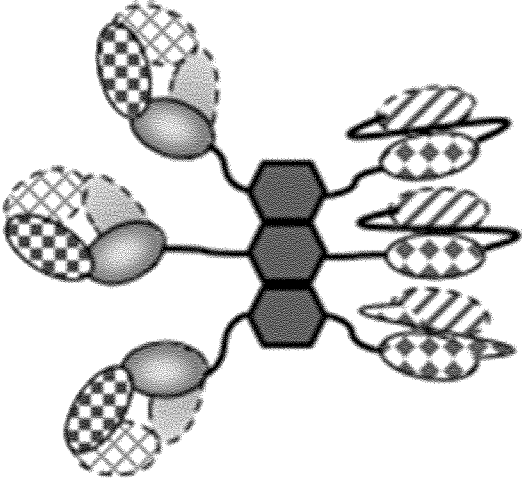
Anti-OX40



Anti-FAP

FIG.2B

Anti-4-1BB



Anti-FAP

Fig. 3

FIG.3A

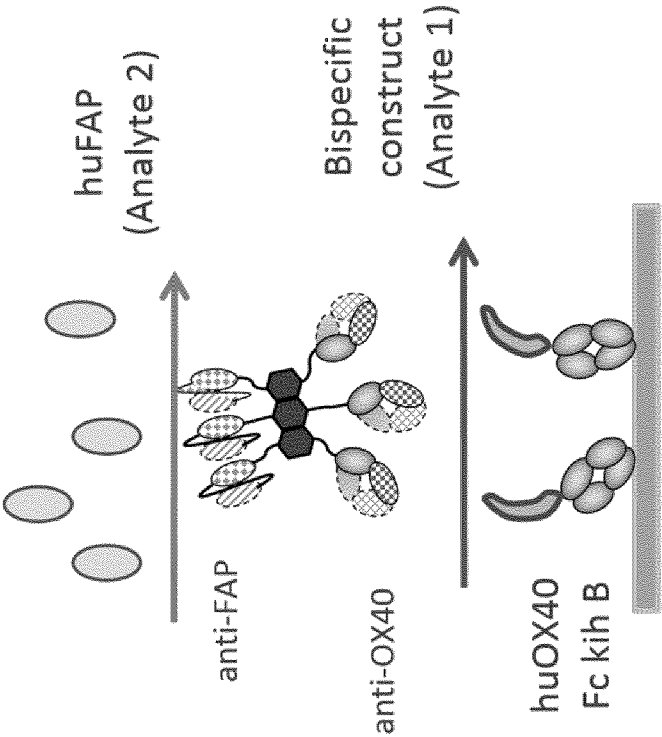


FIG.3B

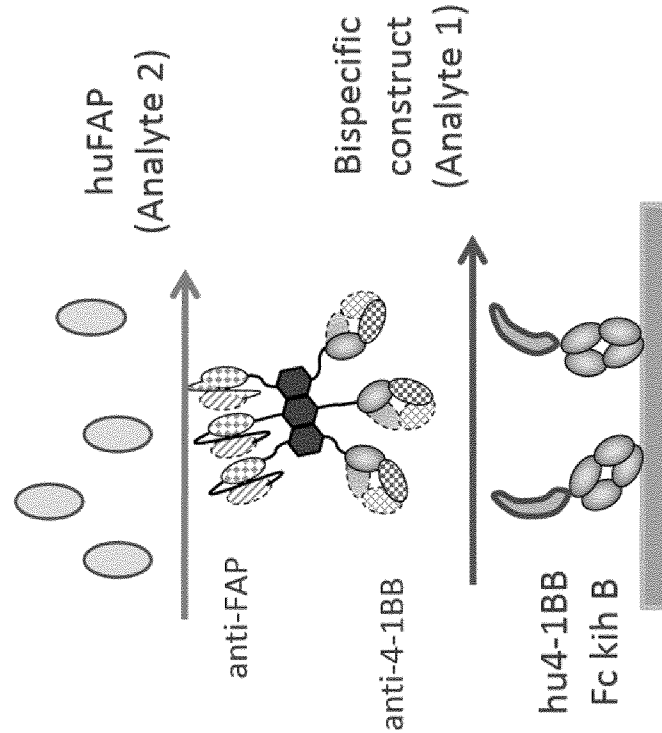


Fig. 4

FIG.4A

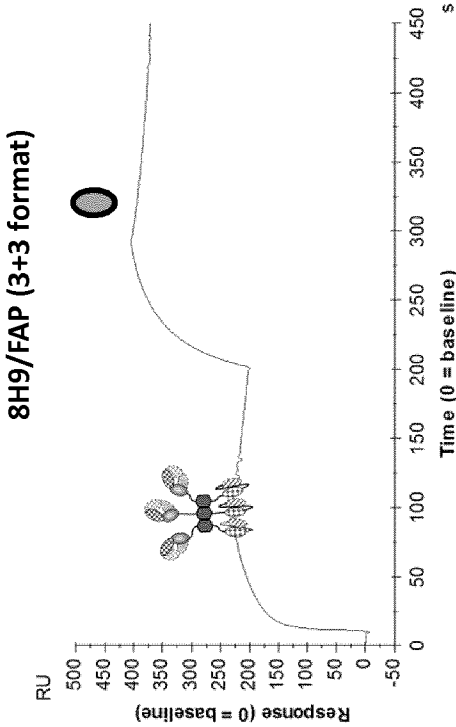


FIG.4B

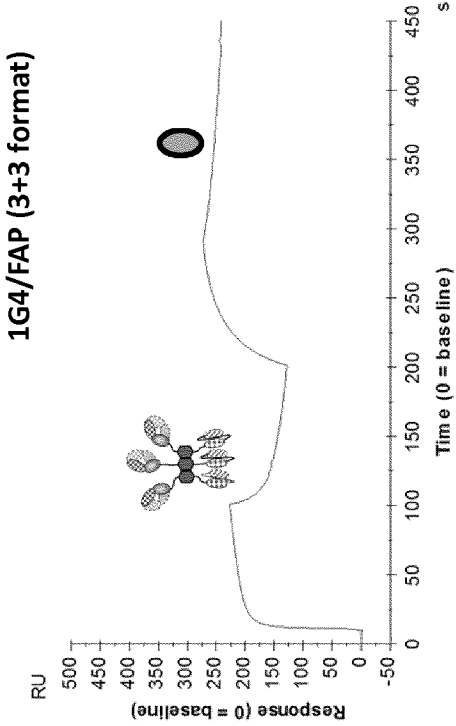


FIG.4C

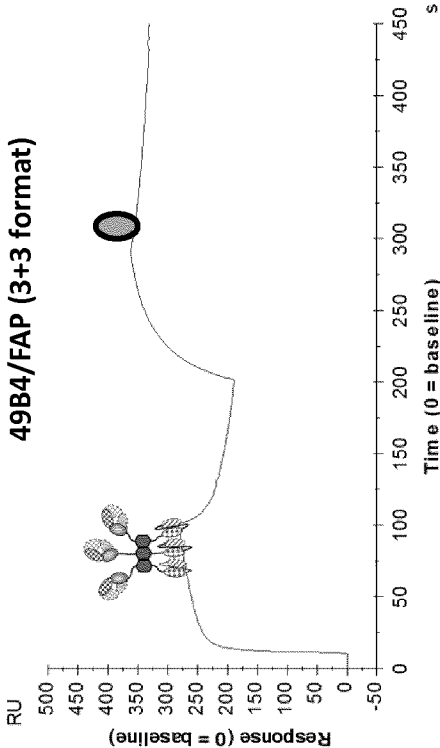


FIG.4D

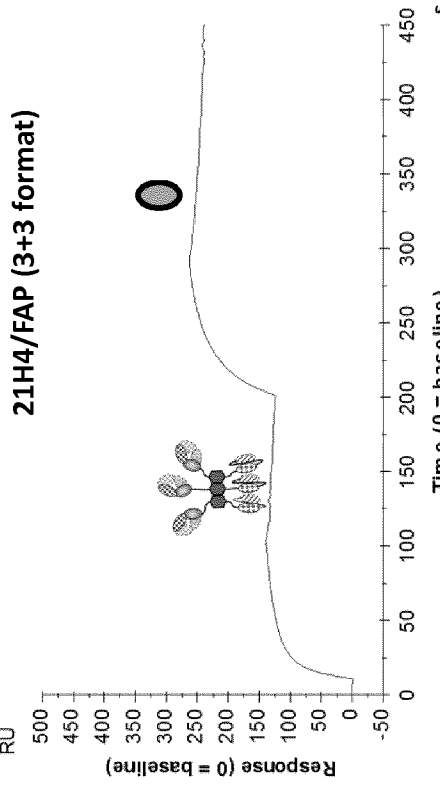


FIG.4E

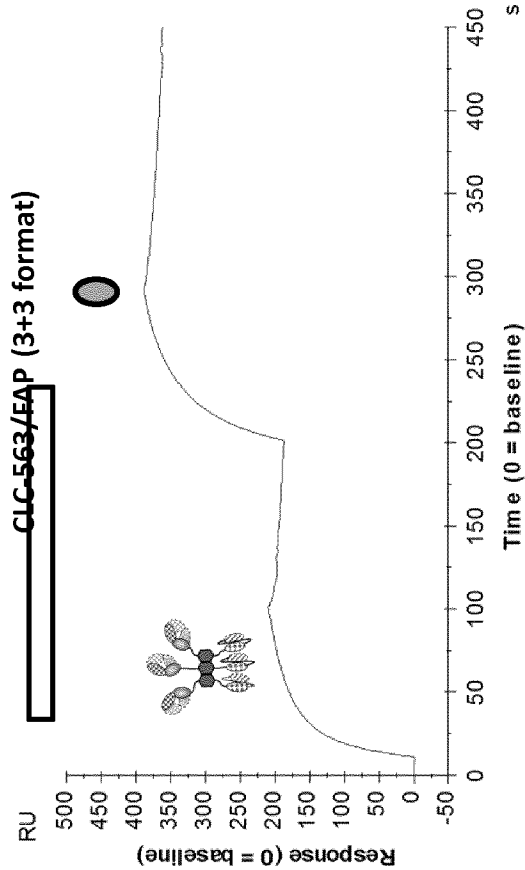


Fig. 5

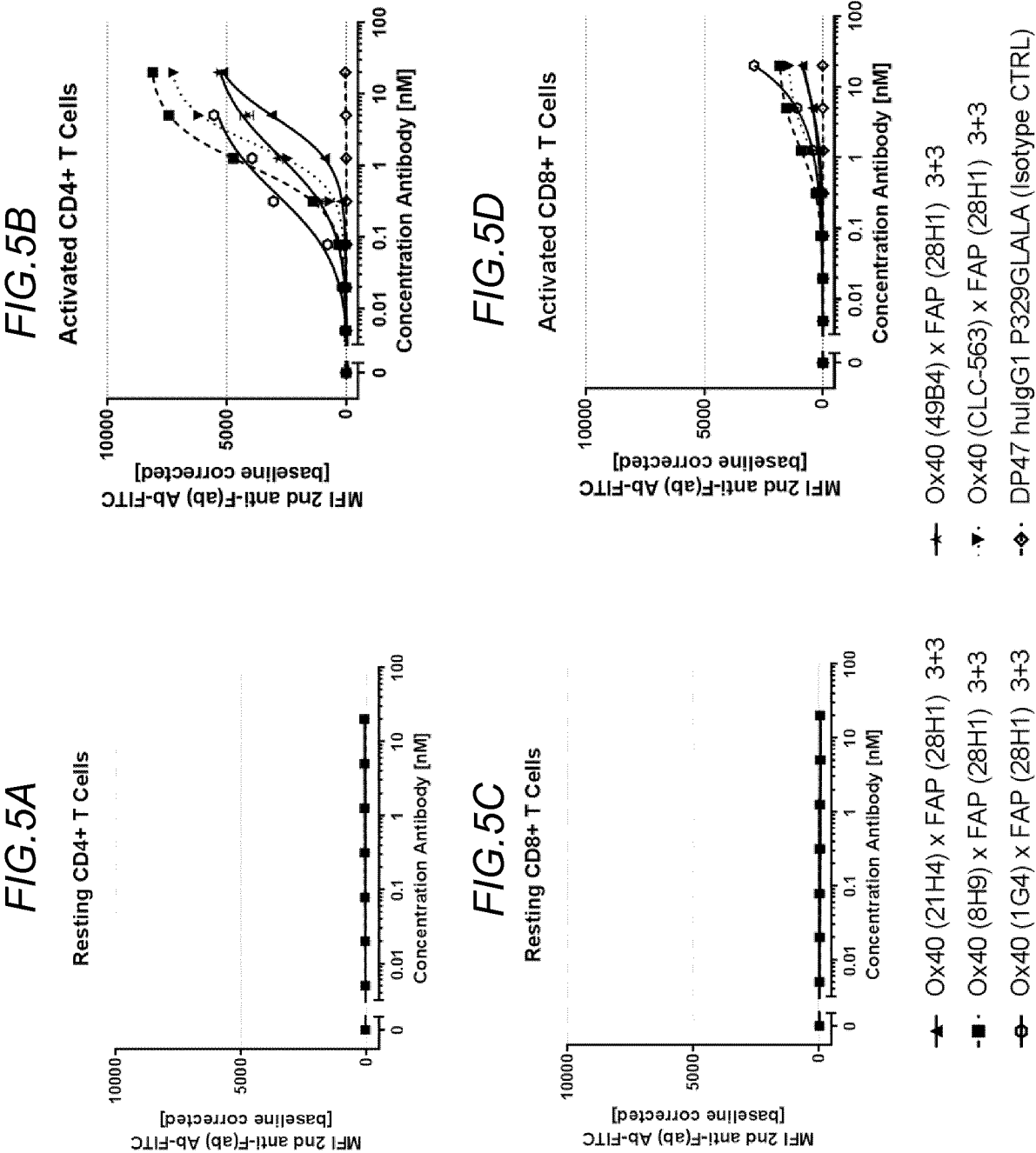


Fig. 6

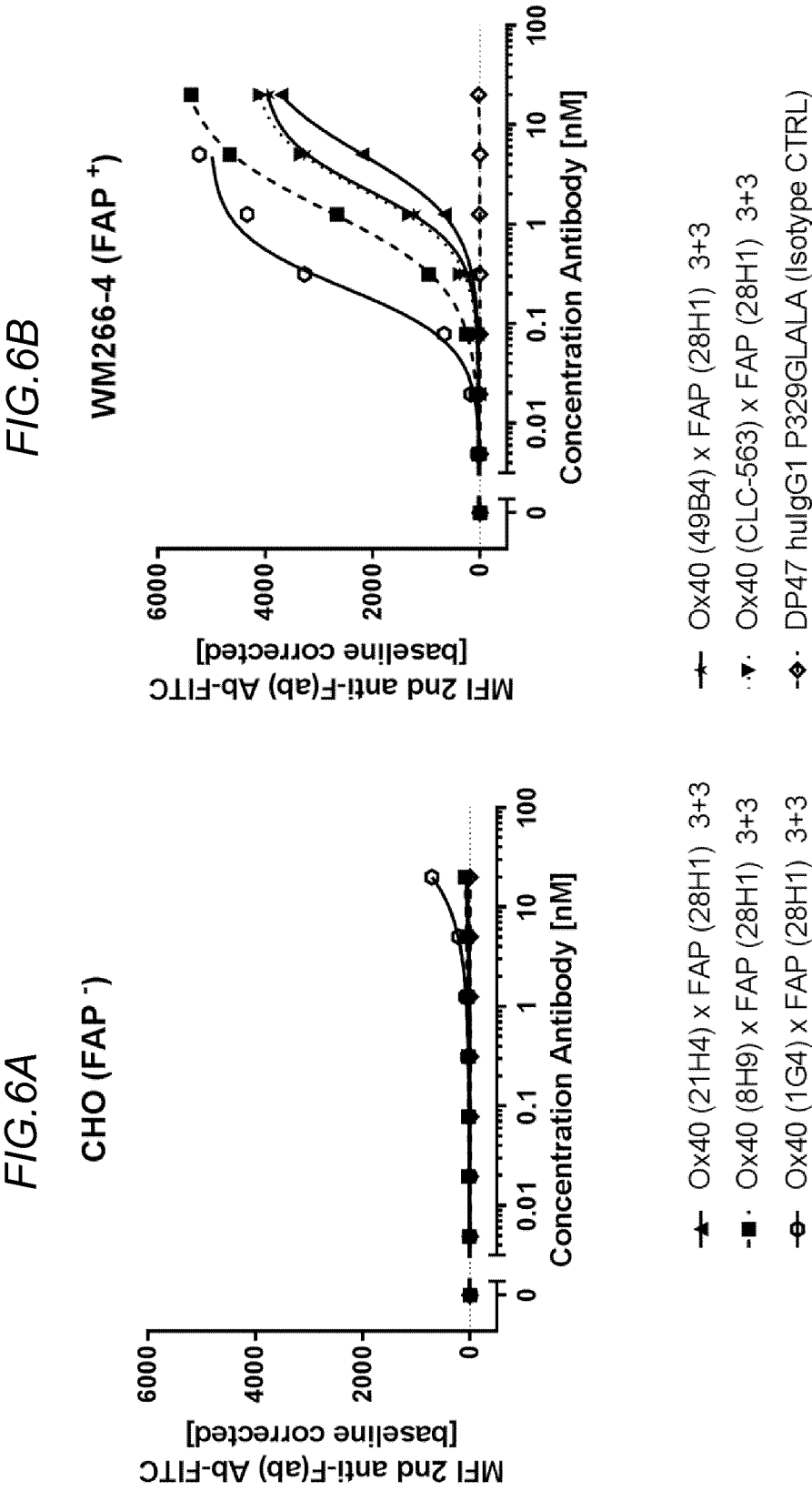


Fig. 7

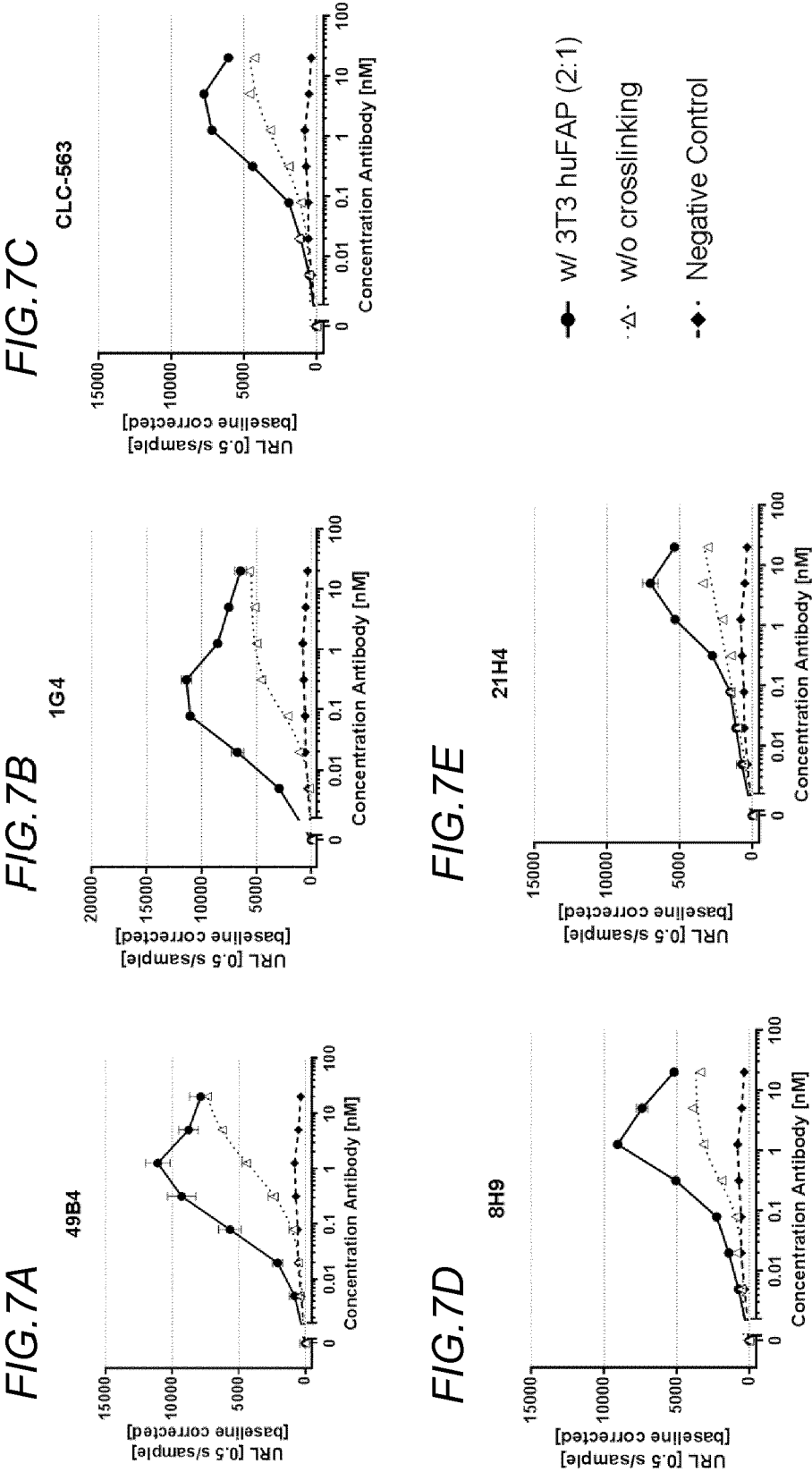


Fig. 8

FIG.8A

Performance targeted

w/ 3T3 FAP (2:1)

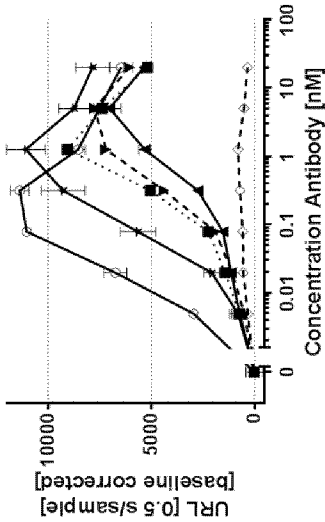


FIG.8B

Performance untargeted

w/o Crosslinking

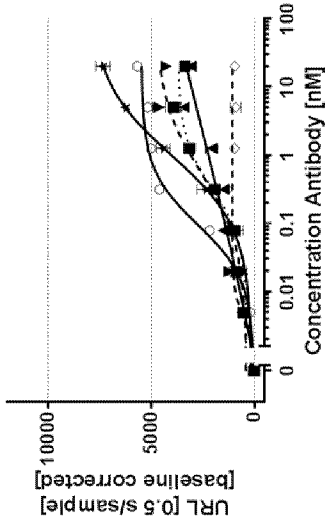
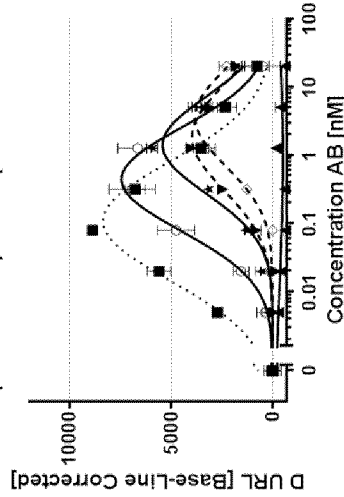


FIG.8C

= Effect gained by targeting

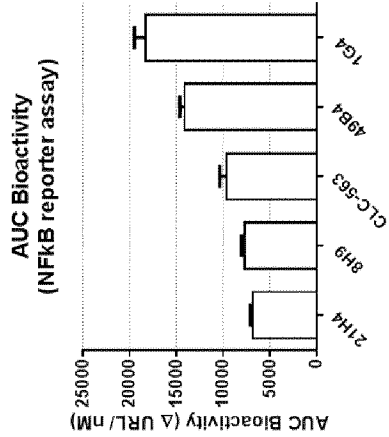
URL (w/3T3 FAP) - URL (w/o crosslinking)



- ▲ Ox40 (21H4) x FAP (28H1) 3+3
- Ox40 (8H9) x FAP (28H1) 3+3
- Ox40 (1G4) x FAP (28H1) 3+3

FIG.8D

Quantified as Area under the curve



- ▲ Ox40 (49B4) x FAP (28H1) 3+3
- ▼ Ox40 (CLC-563) x FAP (28H1) 3+3
- ◆ DP47 huIgG1 P329GLALA (isotype CTRL)

Fig. 9

FIG.9A

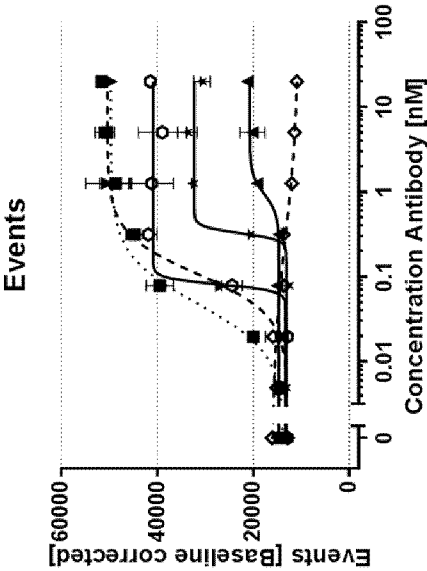


FIG.9B

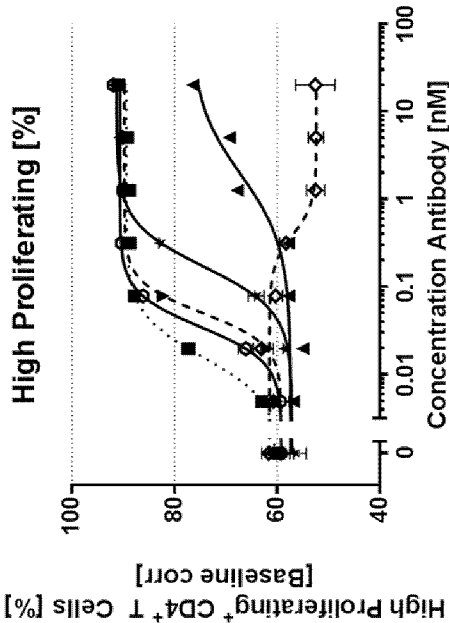


FIG.9C

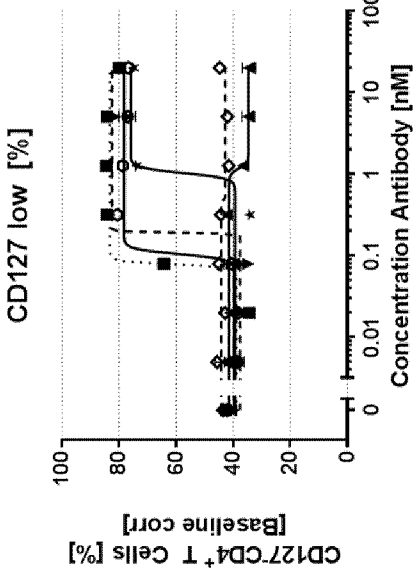
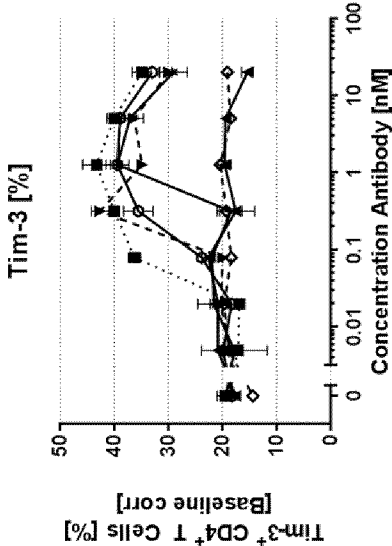


FIG.9D



- △— Ox40 (21H4) x FAP (28H1) 3+3
- Ox40 (8H9) x FAP (28H1) 3+3
- Ox40 (1G4) x FAP (28H1) 3+3

- △— Ox40 (49B4) x FAP (28H1) 3+3
- ▽- Ox40 (CLC-563) x FAP (28H1) 3+3
- ◇- DP47 huIgG1 P329GLALA (Isotype CTRL)

11/17

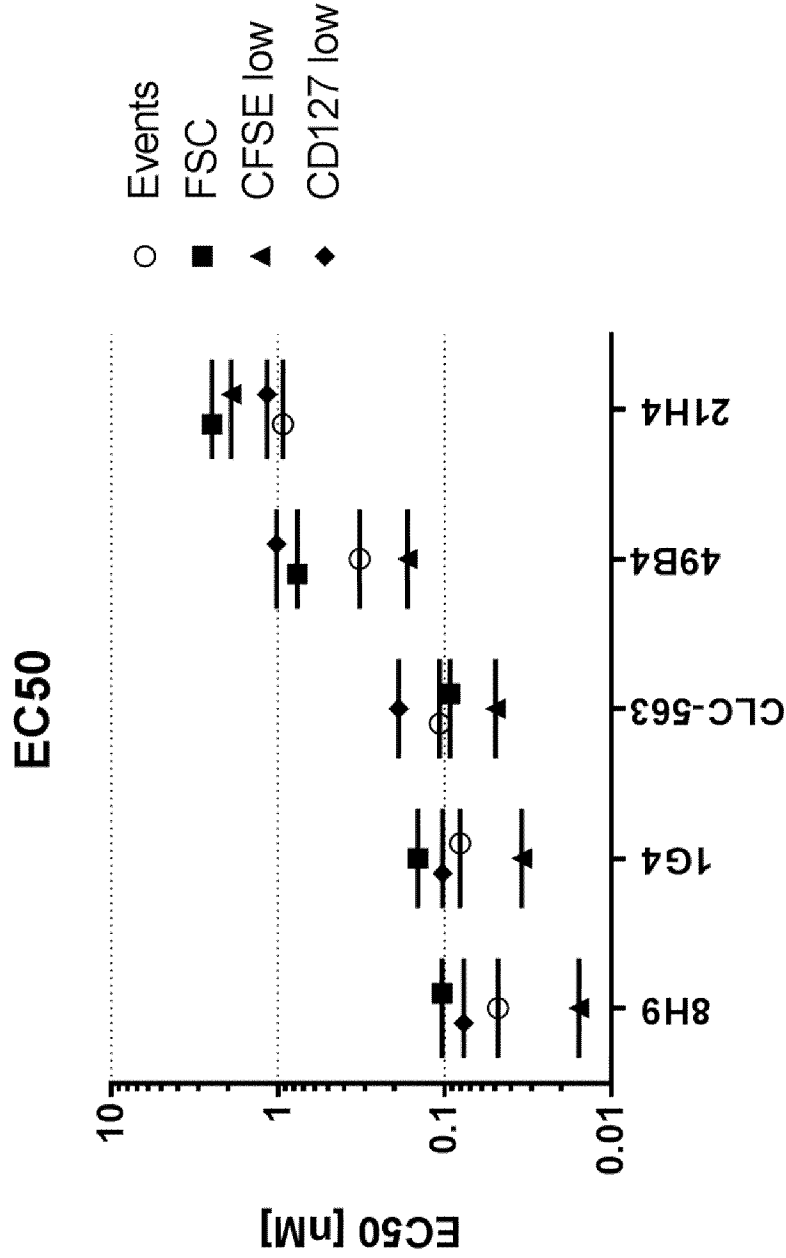


Fig. 10

Fig. 11

FIG. 11A

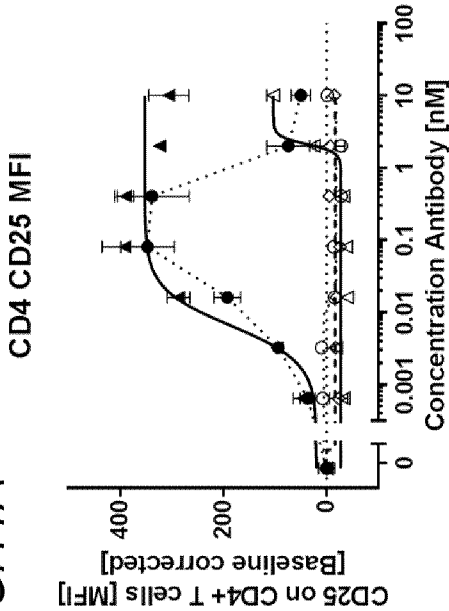


FIG. 11B

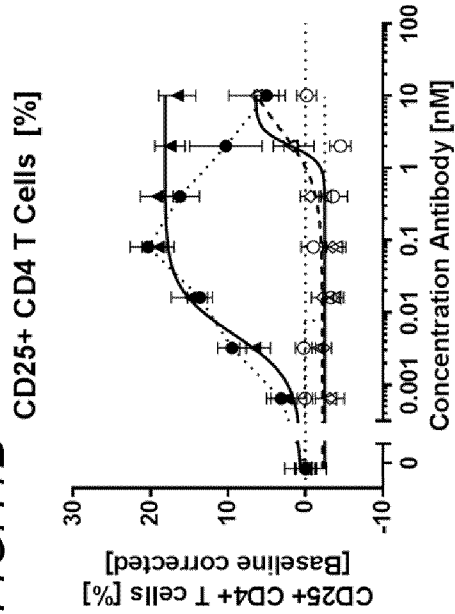


FIG. 11C

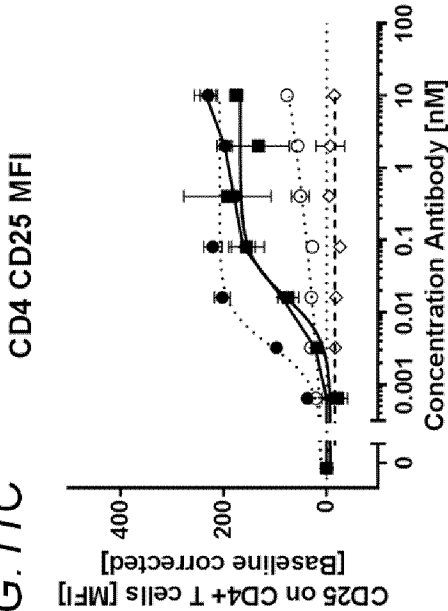


FIG. 11D

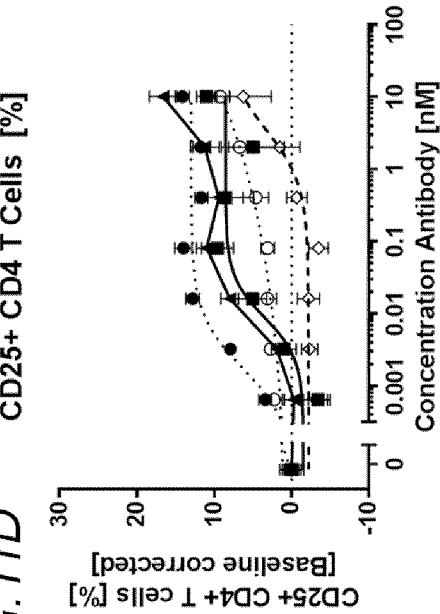
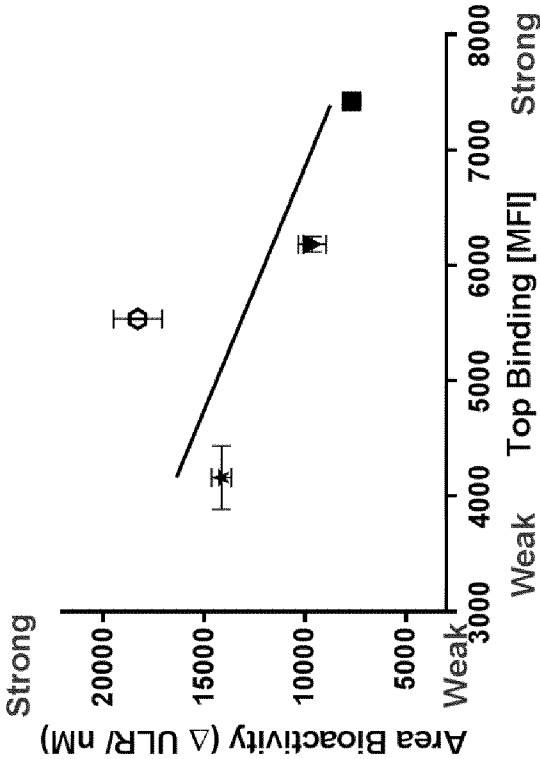


Fig. 12

FIG. 12A

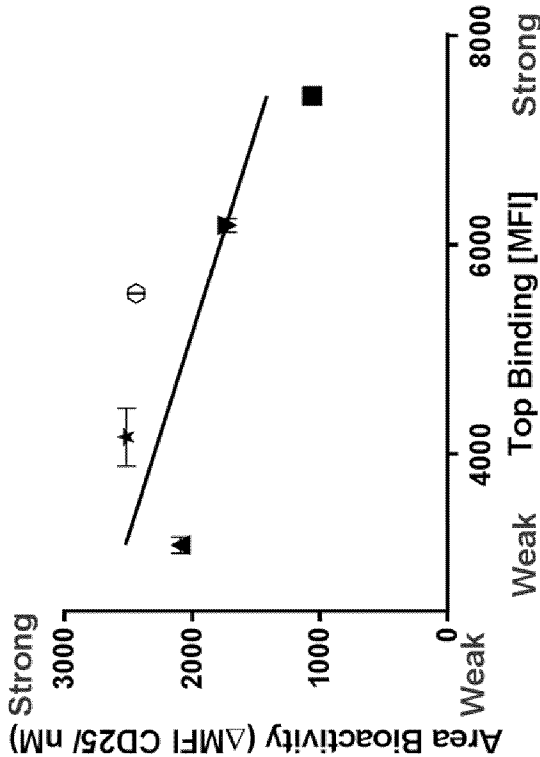
Top Binding
vs AuC Bioactivity
(NfKB reporter assay)



- ▲ Ox40 (21H4) x FAP (28H1) 3+3
- Ox40 (8H9) x FAP (28H1) 3+3
- Ox40 (1G4) x FAP (28H1) 3+3

FIG. 12B

Top Binding
vs Area Bioactivity
(huPBMC assay)



- ▲ Ox40 (49B4) x FAP (28H1) 3+3
- ▼ Ox40 (CLC-563) x FAP (28H1) 3+3
- ◆ DP47 huIgG1 P329GLALA (Isotype CTRL)

Fig. 13

FIG.13A

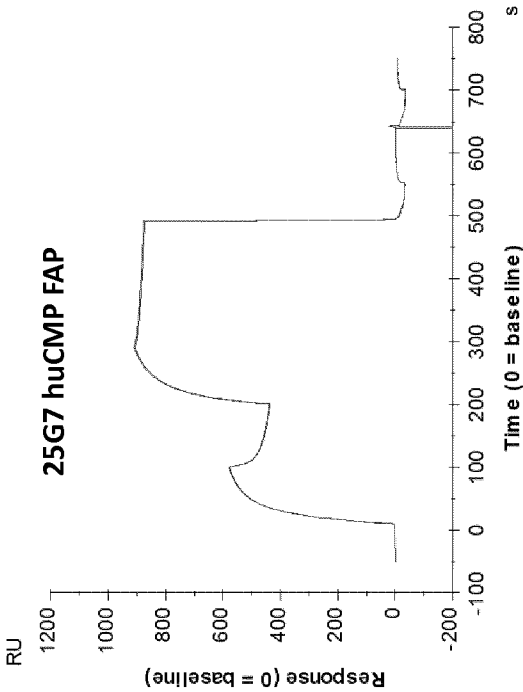


FIG.13B

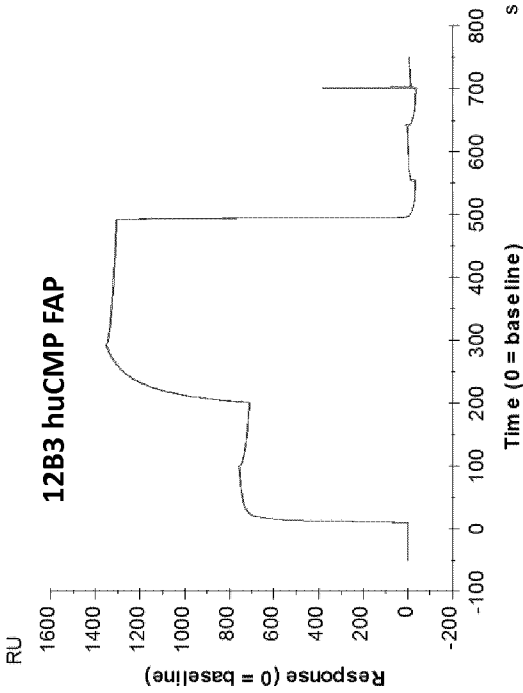


FIG.13C

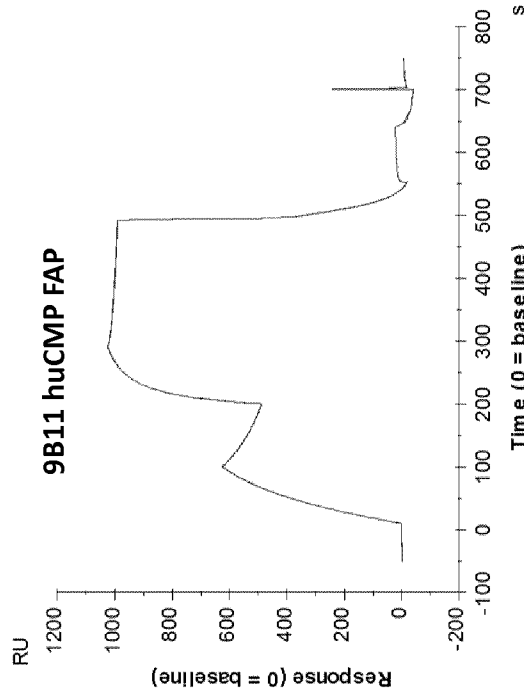


Fig. 14

FIG.14A

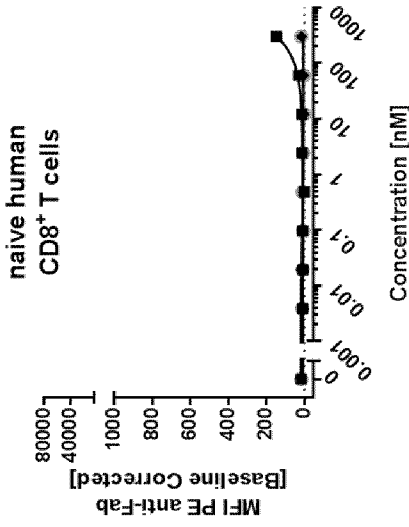


FIG.14B

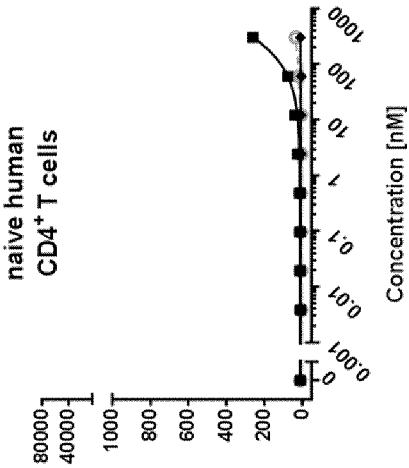


FIG.14C

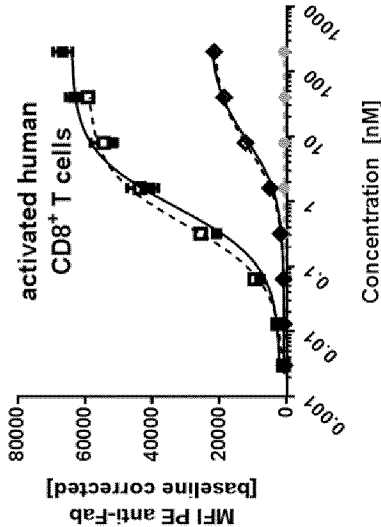


FIG.14D

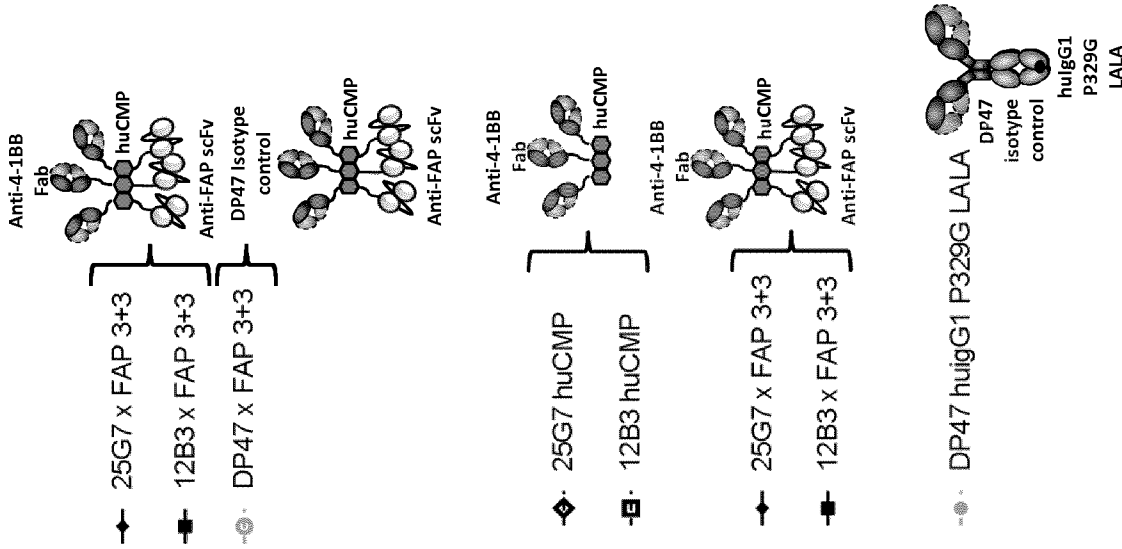
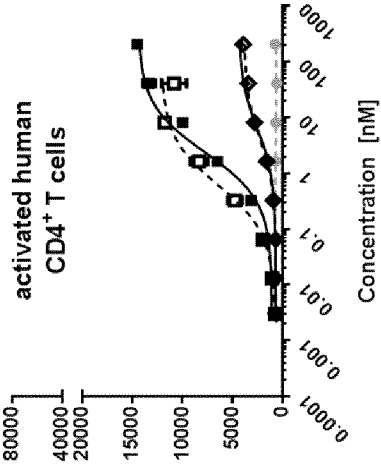


Fig. 15

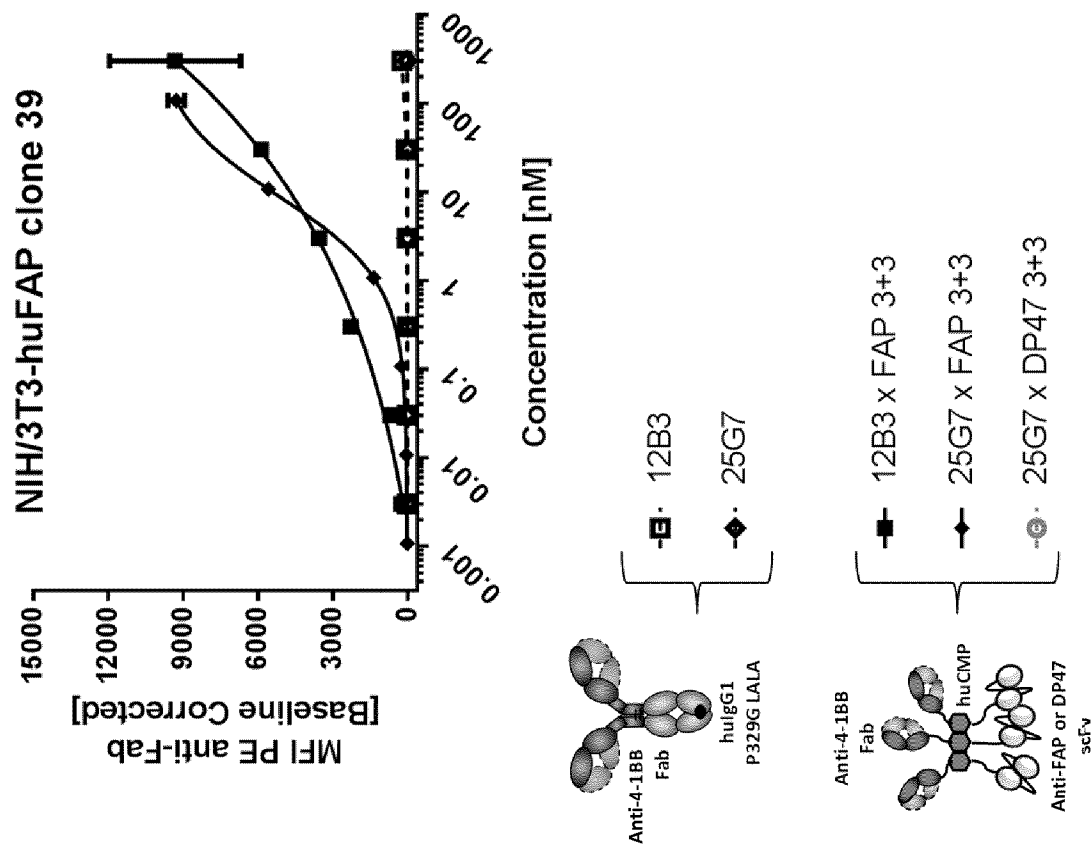


Fig. 16

FIG. 16A

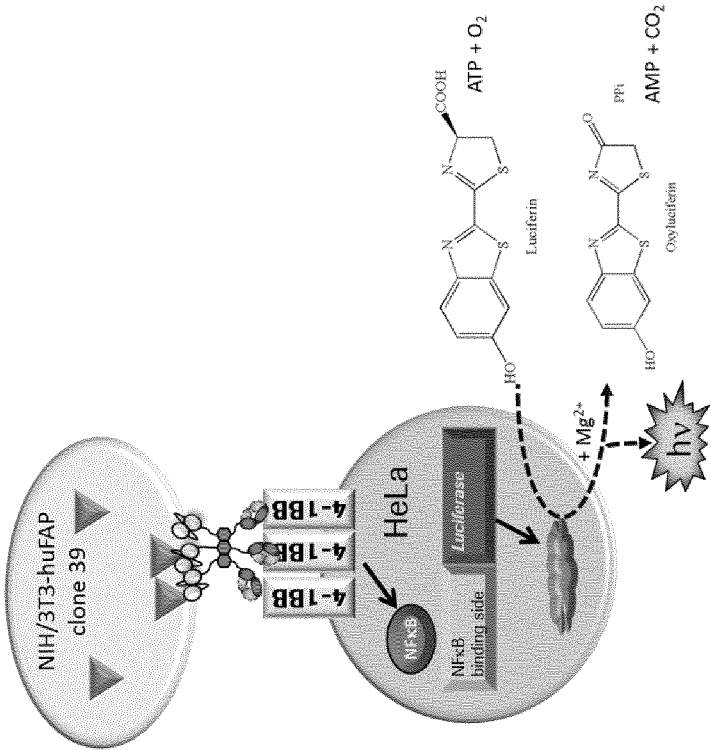
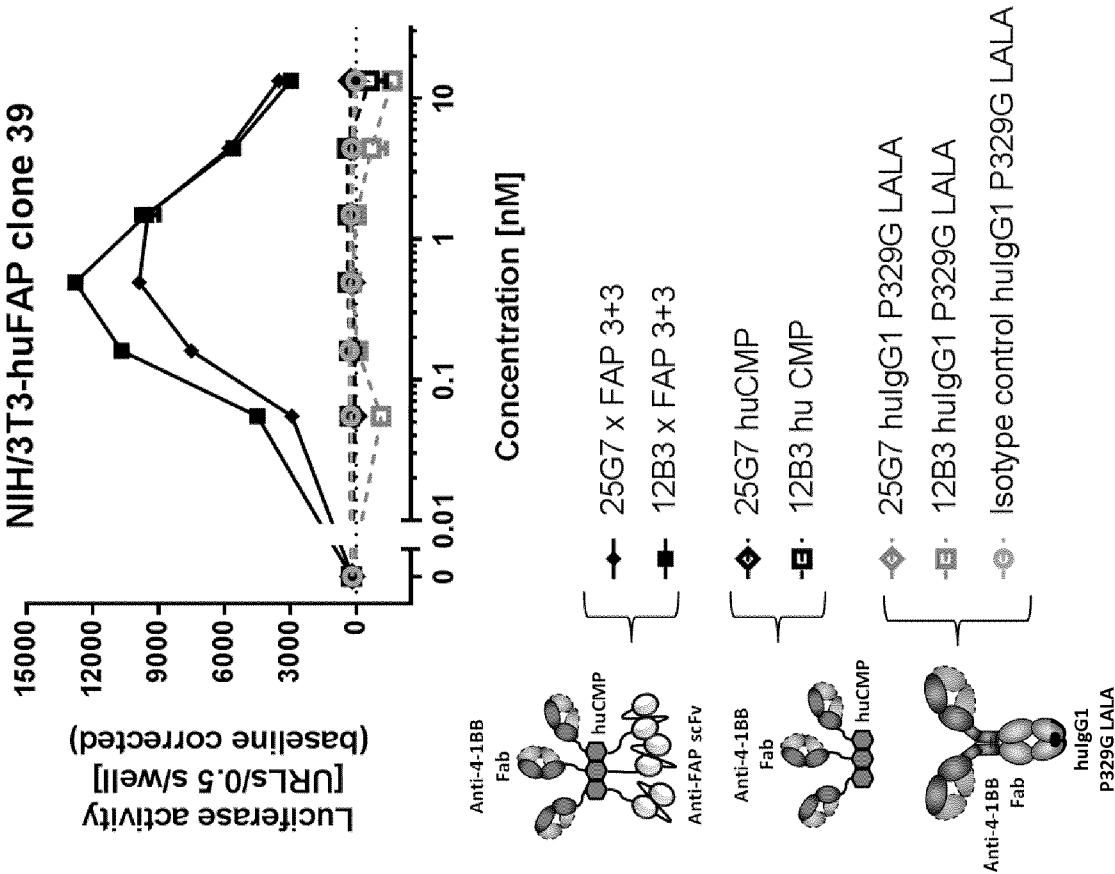


FIG. 16B



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/057767

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/705 A61K38/17 A61K39/395 C07K16/28 C07K16/30 C12N15/62 ADD. According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K A61K C12N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category*</th> <th style="width: 70%;">Citation of document, with indication, where appropriate, of the relevant passages</th> <th style="width: 20%;">Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">X Y</td> <td style="vertical-align: top;"> WO 2017/025610 A1 (MEDIMMUNE LTD [GB]) 16 February 2017 (2017-02-16) abstract paragraphs [0002], [0003] - [0018], [0095], [0098], [0100] - [0105], [0124], [0173], [0178] - [0180] ----- </td> <td style="vertical-align: top;"> 1-5, 16-25 6-12 </td> </tr> <tr> <td style="vertical-align: top;">X Y</td> <td style="vertical-align: top;"> WO 2015/183902 A1 (MEDIMMUNE LLC [US]) 3 December 2015 (2015-12-03) abstract paragraphs [0005] - [0008], [0012], [0029] - [0032], [0070], [0128], [0129] ----- </td> <td style="vertical-align: top;"> 1-5, 16-25 6-12 </td> </tr> <tr> <td style="vertical-align: top;">Y</td> <td style="vertical-align: top;"> WO 2014/180754 A1 (HOFFMANN LA ROCHE [CH]; HOFFMANN LA ROCHE [US]) 13 November 2014 (2014-11-13) abstract page 4, line 21 - page 6, line 30 ----- -/-- </td> <td style="vertical-align: top;"> 1-25 </td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X Y	WO 2017/025610 A1 (MEDIMMUNE LTD [GB]) 16 February 2017 (2017-02-16) abstract paragraphs [0002], [0003] - [0018], [0095], [0098], [0100] - [0105], [0124], [0173], [0178] - [0180] -----	1-5, 16-25 6-12	X Y	WO 2015/183902 A1 (MEDIMMUNE LLC [US]) 3 December 2015 (2015-12-03) abstract paragraphs [0005] - [0008], [0012], [0029] - [0032], [0070], [0128], [0129] -----	1-5, 16-25 6-12	Y	WO 2014/180754 A1 (HOFFMANN LA ROCHE [CH]; HOFFMANN LA ROCHE [US]) 13 November 2014 (2014-11-13) abstract page 4, line 21 - page 6, line 30 ----- -/--	1-25
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X Y	WO 2017/025610 A1 (MEDIMMUNE LTD [GB]) 16 February 2017 (2017-02-16) abstract paragraphs [0002], [0003] - [0018], [0095], [0098], [0100] - [0105], [0124], [0173], [0178] - [0180] -----	1-5, 16-25 6-12												
X Y	WO 2015/183902 A1 (MEDIMMUNE LLC [US]) 3 December 2015 (2015-12-03) abstract paragraphs [0005] - [0008], [0012], [0029] - [0032], [0070], [0128], [0129] -----	1-5, 16-25 6-12												
Y	WO 2014/180754 A1 (HOFFMANN LA ROCHE [CH]; HOFFMANN LA ROCHE [US]) 13 November 2014 (2014-11-13) abstract page 4, line 21 - page 6, line 30 ----- -/--	1-25												
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Date of the actual completion of the international search 9 May 2018		Date of mailing of the international search report 29/05/2018												
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Chavanne, Franz												

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/057767

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2016/156291 A1 (F HOFFMANN-LA ROCHE AG [CH]; HOFFMANN-LA ROCHE INC [US]) 6 October 2016 (2016-10-06) abstract page 1, lines 1-7 page 4, line 13 - page 13, line 19 -----	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/057767

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017025610 A1	16-02-2017	AU 2016306625 A1	29-03-2018
		CA 2994346 A1	16-02-2017
		KR 20180033588 A	03-04-2018
		TW 201718632 A	01-06-2017
		US 2017073386 A1	16-03-2017
		WO 2017025610 A1	16-02-2017
WO 2015183902 A1	03-12-2015	CN 106456734 A	22-02-2017
		EP 3148573 A1	05-04-2017
		GB 2532817 A	01-06-2016
		JP 2017519494 A	20-07-2017
		TW 201623327 A	01-07-2016
		US 2016024176 A1	28-01-2016
		WO 2015183902 A1	03-12-2015
WO 2014180754 A1	13-11-2014	CA 2907597 A1	13-11-2014
		CN 105377893 A	02-03-2016
		EP 2994487 A1	16-03-2016
		HK 1222182 A1	23-06-2017
		JP 2016520055 A	11-07-2016
		KR 20160005345 A	14-01-2016
		RU 2015152077 A	13-06-2017
		US 2016159917 A1	09-06-2016
		WO 2014180754 A1	13-11-2014
WO 2016156291 A1	06-10-2016	CN 107207579 A	26-09-2017
		EP 3277305 A1	07-02-2018
		US 2016340399 A1	24-11-2016
		WO 2016156291 A1	06-10-2016