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(54) **Title:** PHARMACEUTICAL COMPOSITIONS COMPRISING BISPECIFIC ANTIBODIES BINDING TO B7H4 and CD3

(57) **Abstract:** The present invention relates to pharmaceutical compositions and unit dosage forms comprising antibodies binding to B7H4 and CD3. The invention further provides a use of the pharmaceutical compositions and unit dosage forms for therapeutic and diagnostic procedures, in particular in cancer therapy.

PHARMACEUTICAL COMPOSITIONS COMPRISING BISPECIFIC ANTIBODIES BINDING TO B7H4 and CD3

Field of Invention

The present invention relates to pharmaceutical compositions and unit dosage forms of bispecific antibodies binding to B7H4 and CD3, and to the use of such pharmaceutical compositions or unit dosage forms.

Introduction

B7H4 (B7-H4, V-set domain containing T cell activation inhibitor 1 or VTCN1) is a member of the B7 family of proteins, which family comprises cell-surface protein ligands that bind to receptors on lymphocytes. The B7 family plays an important role in the regulation of immune responses. B7H4 negatively regulates T cell-mediated immune responses by inhibiting T cell activation, proliferation, cytokine production and cytotoxic activity (Prasad et al., 2003, Immunity 18: 863-873). B7H4 is a type I transmembrane protein that includes a short intracellular domain, a hydrophobic transmembrane domain, and an extracellular domain with an IgV-and an IgC-like domain with four conserved cysteine residues and seven sites for N-linked glycosylation. (Sica et al., 2003, Immunity 18: 849-861). To date, no receptor for B7H4 has been identified.

In normal adult tissue, B7H4 expression is very limited, whereas B7H4 expression is found on tumor cells in numerous cancer tissues (Kaur and Janakiram, 2019, ESMO Open 4:e000554). In cancer, B7H4 expression is correlated with advanced stages of cancer, poor prognosis, and decreased overall patient survival.

Hence, targeting of B7H4 has been proposed for the treatment of cancer (Podojil and Miller, Immunological Reviews, 2017: 276; 40-51). Currently, B7H4 binding antibodies are in development for cancer therapy. For example, FPA150 is an afucosylated human antibody that relieves the B7H4-mediated suppression of T cell activation and exhibits antibody dependent cellular cytotoxicity (ADCC) activity (Wainberg et al., 2019, Annals of Oncology 30, Suppl. 5, v489 (1198P). It is currently in early clinical trials as a monotherapy or in combination with pembrolizumab in advanced solid tumors.

Efforts to target T cells to B7H4 have also been made. A B7H4/CD3-bispecific single chain antibody, Fab scFv, was made based on the Fab and single-chain variable fragments (scFv) structure of a mouse anti-human B7H4 antibody and a mouse anti-human CD3 antibody (Iizuka et al., 2019, Clin Cancer Res 25: 2925-2934). Smith et al. have described engineered T cells with B7H4-specific chimeric antigen receptors (CARs) that displayed anti-tumor activity against B7H4-positive human ovarian tumor

xenografts, but which also showed multi-organ lymphocytic infiltration and lethal toxicity (Smith et al. 2016, Molecular Therapy, Vol.24 Iss. 11 pp 1987-99) in mice.

While there has been some progress made, there is a continued need for the development of antibody-based cancer therapy targeting B7H4 that is efficacious and/or safe for human use. There is
5 also a need for pharmaceutically acceptable formulations of antibodies for use in such therapy.

Summary of Invention

It is an object of the present invention to provide novel pharmaceutical compositions of antibodies comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region that binds to CD3, such as human CD3ε (epsilon). It is a further object to provide pharmaceutical
10 compositions of the antibodies which formulations are stable over a broad range of antibody concentrations and/or temperatures. It is a further object to provide pharmaceutical compositions of the antibodies which formulations are stable over a period of at least 3 months, or even longer, such as at least 6 month or at least 12 months. It is a further object of the present invention to provide pharmaceutical formulations of the antibodies which are well tolerated for IV infusion. The antigen-
15 binding regions of such antibodies comprise at least human framework regions, such as e.g FR1, FR2, FR3 and FR4. Most preferred is that all framework regions are human. Such antigen-binding regions are humanized and/or human antigen-binding regions. These pharmaceutical compositions are useful in the treatment of conditions wherein specific targeting and T cell mediated killing of B7H4 expressing cells is desired, e.g. in conditions such as cancer. Preferably, the pharmaceutical composition is
20 suitable for human use, e.g. in a medical treatment. Cancers that may be suitable for treatment are solid tumors. Said B7H4 expression, and T cell mediated killing, e.g. in cancer cells, may range in accordance with the invention from relatively low expression of B7H4, such as in MCF-7 cells, to relatively high expression of B7H4, such as in SK-BR3 cells, as shown e.g. in example 12. More preferably such bispecific antibodies have substitutions within the constant region that renders the Fc
25 region, if present, inert. In a preferred embodiment, the pharmaceutical composition of the present invention is suitable both for IV administration.

In a first aspect, the present invention provides a pharmaceutical composition comprising a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy
30 and light chain variable regions, wherein said heavy and light chain variable regions are humanized and/or human, and b) a buffering agent, wherein the pH of the composition is from 4.0 to 8.0, preferably 4.5 to 6.5, most preferably 5.0 to 6.0. The buffering agent is preferably selected from the

group consisting of histidine, glutamate, and mixtures thereof, and the pharmaceutical composition preferably further comprises a non-ionic excipient. It has been found that such pharmaceutical compositions provide a surprisingly high stability of the antibody, such as thermal stability and storage stability, as well as a high degree of solubility.

5 In one embodiment, the pharmaceutical composition of the present invention comprises a bispecific antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region that is capable of binding to CD3, such as human CD3ε (epsilon), wherein the antigen-binding region capable of binding to human B7H4 comprises a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25, 29 or 31 , and a variable light chain 10 region comprising the CDR1, CDR2 and CDR3 of SEQ ID NO. 33 and wherein the antigen-binding region that is capable of binding to CD3 comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of 18, 19 and 21 respectively; and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively. Preferably the CDR regions are determed by the IMGT method described below.

15 In a further aspect, said pharmaceutical compositions in accordance with the present invention are for use in a medical treatment.

Brief description of the figures

Figure 1. Determination of B7H4 domain involved in binding using B7H4-B7H3 chimeric molecules. The B7H4 domain specificity of the B7H4 antibodies was determined using a panel of cells transfected 20 to express human B7H4 (I), human B7H4-B7H3 chimeric molecules B7H3-IgV/B7H4-IgC (II) or B7H4-IgV/B7H3-IgC (III), or human B7H3 (IV). Binding was determined by flow cytometry. A = bsIgG1-huCD3-FEALxB7H4-C4-FEAR; B = bsIgG1-huCD3-FEALxB7H4-C3-FEAR; C = bsIgG1-huCD3-FEALxB7H4-C2-FEAR; D = bsIgG1-huCD3-FEALxB7H4-C1-FEAR; E = IgG1-B7H3-BRCA84D.

Figure 2. Binding of B7H4 antibodies to B7H4, B7H3 or B7H4-B7H3 chimeric molecules. Binding of 25 bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C2-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C3-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR to HEK cells transiently transfected to express human B7H4 or the B7H4-B7H3 chimeric molecules B7H3-IgV/B7H4-IgC or B7H4-IgV/B7H3-IgC was assessed using flow cytometry.

30 **Figure 3. Binding of B7H4 antibodies to B7H4 variants with alanine mutations in the ECD.** Binding was expressed as fold change compared to a reference antibody. Fold change was defined as Log10(Normalized gMFI[ala mutant]/Normalized gMFI[wt]). Residues where the Fold Change in

binding was lower than mean Fold Change − 1.5 x SD were considered 'loss of binding mutants'. Residues with a positive Fold Change in binding are loss of binding residues for the reference antibody. Numbers below the x-axis refer to amino acid positions. (A) Results for C1-N52S, with C2 as reference antibody. (B) Results for C2, with C1-N52S as reference antibody. (C) Results for C3, with C2 as
5 reference antibody.

Figure 4. Binding of B7H4 antibody and CD3xB7H4 bispecific antibody to human and cynomolgus monkey B7H4. Binding of IgG1-B7H4-C1-N52S-FEAR (A) and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (B) to HEK-293F cells transiently transfected with human B7H4 or cynomolgus monkey B7H4 was determined by flow cytometry. Non-transfected HEK-293F cells (C) were used as negative
10 control; for these binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR is shown.

Figure 5. Binding of B7H4 antibody and CD3xB7H4 bispecific antibody to B7H4 from rabbit, rat, mouse, dog and pig. Binding of IgG1-B7H4-C1-N52S-FEAR (A) and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (B) to HEK-293F cells transiently transfected with B7H4 from rabbit, rat, mouse, dog or pig was determined by flow cytometry. Non-transfected HEK-293F cells (C) were used as negative
15 control; for these binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR is shown.

Figure 6. Binding of B7H4 antibodies to HEK-293F cells transiently transfected with B7H4 from different species. Binding of IgG1-B7H4-C1-N52S-FEAR (A), IgG1-B7H4-C3-FEAR (B), IgG1-B7H4-C2-FEAR (C),IgG1-B7H4-C4-FEAR (D), and IgG1-B7H4-C5-FEAR (E) to HEK-293F cells transfected with B7H4 from human, cynomolgus monkey, mouse, rat or pig, or to untransfected HEK-293F cells, was
20 determined by flow cytometry. IgG1-b12 was used as non-binding control antibody (not shown).

Figure 7. Binding of IgG1-B7H4-C1-N52S-FEAR (A) and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (B) to MCF-7 and MDA-MB-468 cells. Binding was determined by flow cytometry. IgG1-b12 (C) and bsIgG1-huCD3-H101G-FEALxb12-FEAR (D) were used as non-binding control antibodies.

**Figure 8. Binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (A) to NIH-OVCAR-3, HCC1954
25 and HeLa cells.** Binding was determined by flow cytometry. BsIgG1-huCD3-H101G-FEALxb12-FEAR (B) was used as non-binding control antibody.

Figure 9. Binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (A) and bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (B) to SK-BR3 and MDA-MB-486 cells. Binding was determined by flow cytometry. bsIgG1-huCD3-FEALxb12-FEAR (C) and bsIgG1-huCD3-H101G-FEALxb12-FEAR (D) were
30 used as non-binding control antibodies.

Figure 10 Binding of various B7H4 antibodies in homodimer and bsAb format to MDA-MB-486 and HCC1954 cells. Binding of IgG1-B7H4-C1-N52S-FEAR (A homodimer), IgG1-B7H4-C2-FEAR (B homodimer), IgG1-B7H4-C3-FEAR (C homodimer), IgG1-B7H4-C4-FEAR (D homodimer), IgG1-B7H4-C5-FEAR (E homodimer), bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (A bsAb), bsIgG1-huCD3-FEALxB7H4-C2-FEAR [MDA-MB-468] or bsIgG1-huCD3-H101G-FEALxB7H4-C2-FEAR [HCC1954] (B bsAb), bsIgG1-huCD3-H101G-FEALxB7H4-C3-FEAR (C bsAb), bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR (D bsAb), and bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR (E bsAb) was determined by flow cytometry. bsIgG1-huCD3-H101G-FEALxb12-FEAR (F bsAb) or IgG1-b12-K409R (F homodimer) was used as a non-binding control antibody.

Figure 11. Induction of T cell mediated cytotoxicity of SK-BR3 cells *in vitro* by CD3xB7H4 bispecific antibodies using purified T cells as effector cells at varying effector to target ratios (E:T). bsIgG1-huCD3-FEALxb12-FEAR was used as non-binding control antibody. A = bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR; B = bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR; C = bsIgG1-huCD3-FEALxb12-FEAR.

Figure 12. Induction of T cell mediated cytotoxicity in various tumor cell lines *in vitro* in the presence of CD3xB7H4 bispecific antibodies with different CD3 arms. bsIgG1-huCD3-FEALxb12-FEAR was used as non-binding control antibody. A = bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR; B = bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR; C = bsIgG1-huCD3-FEALxb12-FEAR, D = bsIgG1-huCD3-H101G-FEALxb12-FEAR.

Figure 13. B7H4 expression levels and IC50 of T cell-mediated tumor cell killing. (A) Quantitative flow cytometric analysis of B7H4 expression levels on tumor cell lines. Shown are individual measurements (dots), geometric means (bars) and standard deviation (error bars). sABC = specific antibody binding capacity. (B) IC50 of T cell-mediated tumor cell killing in the presence of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (I) or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (II) for the different tumor cell lines. Each dot represents an experiment performed with an individual T cell donor (4-6 donors per cell line), horizontal lines indicate median. Cell lines are ranked according to B7H4 expression level.

Figure 14. T cell activation by B7H4 bispecific antibodies in T cell-tumor cell co-cultures. (A) T cell activation (% of CD69 on CD8+ cells) in the presence of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (I) or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (II) for various B7H4-positive tumor cell lines, determined by flow cytometry. (B) EC50 of T cell activation, using T cells derived from 3-5 donors, for each of the target cell lines. Each dot represents an experiment performed with an individual T cell donor; horizontal lines indicate geometric mean.

Figure 15. IFNγ in the supernatant of T cell-tumor cell co-cultures at EC50, EC90 and EC99 for bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (A) and bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (B) using T cells from 3-4 donors, determined by a multiplex U-plex assay. Shown are individual measurements (dots), geometric means (horizontal lines) and standard deviation (error bars).

5 **Figure 16. IL-6 and MCP-1 levels in the plasma of cynomolgus monkeys treated with single dose IV infusion of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (A) or bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (B).**

Figure 17. Mean plasma concentration-time profiles following a single IV infusion of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (A) or bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (B).

10 **Figure 18. B7H4 mRNA expression levels in a range of primary solid tumors.** B7H4 mRNA levels were extracted from the Omicsoft TCGA database and visualized using Oncoland software. Indications are ranked according to median of the B7H4 mRNA expression. THYM = thymoma, UVM = uveal melanoma, PCPG = pheochromocytoma and paraganglioma, ACC = adrenocortical carcinoma, MESO = mesothelioma, SKCM = skin cutaneous melanoma, READ = rectum adenocarcinoma, COAD = colon
15 adenocarcinoma, GMB = glioblastoma multiforme, SARC = sarcoma, LIHC = liver hepatocellular carcinoma, LGG = brain lower grade glioma, KIRC = kidney renal clear cell carcinoma, TGCT = testicular germ cell tumors, KICH = kidney chromophobe, STAD = stomach adenocarcinoma, THCA = thyroid carcinoma, HNSC = head and neck squamous cell carcinoma, PRAD = prostate adenocarcinoma, LUAD = lung adenocarcinoma, ESCA = esophageal carcinoma, CESC = cervical squamous cell carcinoma and
20 endocervical adenocarcinoma, KIRP = kidney renal papillary cell carcinoma, UCS = uterine carcinosarcoma, BLCA = bladder urothelial carcinoma, PAAD = pancreatic adenocarcinoma, LUSC = lung squamous cell carcinoma, BRCA = breast invasive carcinoma, UCEC = uterine corpus endometrial carcinoma, OV = ovarian serous cystadenocarcinoma and CHOL = cholangiocarcinoma.

25

TABLE 1 – Amino acid and nucleic acid sequence

SEQ ID NO:	Reference	Domain	Sequence
1	Human B7H4	ORF	MASLGQILFWSIISIIIILAGAIALIIGFGISGRHSITVTTVAS AGNIGEDGILSCTFEPDIKLSDIVIQWLKEGVLGLVHEFK EGKDELSEQDEMFRGRTAVFADQVIVGNASLRLKNVQ LTDAGTYKCYIITSKGKGNANLEYKTGAFSMPEVNVDY NASSETLRCEAPRWFPQPTVVWASQVDQGANFSEVS NTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIAK ATGDIKVTESEIKRRSHLQLLNSKASLCVSSFFAISWALLP LSPYLMLK
2	Macaca fascicularis B7H4 transcript 1	ORF	MASLGQILFWSIISIIFILAGAIALIIGFGISGRHSITVTTVA SAGNIGEDGILSCTFEPDIKLSDIVIQWLKEGVIGLVHEF KEGKDELSEQDEMFRGRTAVFADQVIVGNASLRLKNV QLTDAGTYKCYIITSKGKGNANLEYKTGAFSMPEVNVD YNASSETLRCEAPRWFPQPTVVWASQVDQGANFSEV SNTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIAK ATGDIKVTESEIKRRSHLQLLNSKASLCVSSFLAISWALLP LAPYLMLK
3	Canis familiaris B7H4	ORF	MASPGQNIFWSIISVIIILAGAIALIIGFGISGRHSITVTTLT SAGNIGEDGILSCTFEPDIKLSDIVIQWLKEGVMGLVHE FKEGKDDLSDQDEMFRGRTAVFADQVIGGNASLRLKN VQLTDAGTYKCYIITSKGKGNANLEYKTGAFSIPEVNVD YNASSENLRCEAPRWFPQPTVVWASQADQGANFSEV FNTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIAK ATGDIKVTDSEIKRRSHLQLLNSKASLGVSSFFAISWVLL PLSSYLMLK
4	Oryctolagus cuniculus B7H4	ORF	MASLGQIIFWSIISIIIILAGAIALIIGFGISGRHSITVTTLTS AGNIGEDGILSCTFEPDIRLSDIVIQWLKEGVVGLVHEFK EGKDDLSDQDEMFRGRTAVFTDQVIVGNASLRLKNVQ LTDAGTYKCYIITSKGKGNANLEYKTGAFSMPEVNLDY NASSESLRCEAPRWFPQPTVVWASQVDQGANFSEVS NTSFELNSENVTMKVVSVLYNVTVNNTYSCMIENDIAK ATGDIKVTDSEIKRRSSLQLLNSRAAPSVSPRSAVGWLL LPLSSYVMLK
5	Rattus norvegicus B7H4	ORF	MASLGQIIFWSIINVIIILAGAIVLIIGFGISGKHFITVTTFT SAGNIGEDGTLSCTFEPDIKLNGIVIQWLKEGIKGLVHEF KEGKDDLSQQHEMFRGRTAVFADQVVVGNASLRLKN VQLTDAGTYTCYIHTSKGKGNANLEYKTGAFSMPEINV DYNASSESLRCEAPRWFPQPTVAWASQVDQGANFSE VSNTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIA KATGDIKVTDSEVKRRSQLELLNSGPSPCVSSVSAAGW ALLSLSCCLMLR

6	Mus musculus B7H4	ORF	MASLGQIIFWSIINIIIILAGAIALIIGFGISGKHFITVTTFTS AGNIGEDGTLSCTFEPDIKLNGIVIQWLKEGIKGLVHEFK EGKDDLSQQHEMFRGRTAVFADQVVVGNASLRLKNV QLTDAGTYTCYIRTSKGKGNANLEYKTGAFSMPEINVD YNASSESLRCEAPRWFPQPTVAWASQVDQGANFSEV SNTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIAK ATGDIKVTDSEVKRRSQLQLLNSGPSPCVFSSAFVAGW ALLSLSCCLMLR
7	Sus scrofa B7H4	ORF	MASLGQVVFWSIISIIIILAGAIAFIIGFGISGRHSITVTTLT SAGNIGEDGILSCTFEPDIKLSDIVIQWLKEGVTGLVHEF KKGKDDLSDQDEMFRGRTAVFADQVIVGNASLRLKNV QLTDAGTYKCYIITSKGKGNAKLEYKTGAFSIPEVNVDS NASSESLRCEAPRWFPQPTVVWASQVDQGANFSEVS NTSFELNPENVTMKVVSVLYNVTINTTYSCMIENDIAKA TGDIRVTDSEIKRQSHLQLLNSKASLCLSSFVAISWVLLP LCPYLMLK
8	Kozak		GCCGCCACC
9	B7H3	ORF	MLRRRGSPGMGVHVGAALGALWFCLTGALEVQVPED PVVALVGTDATLCCSFSPEPGFSLAQLNLIWQLTDTKQL VHSFAEGQDQGSAYANRTALFPDLLAQGNASLRLQRV RVADEGSFTCFVSIRDFGSAAVSLQVAAPYSKPSMTLEP NKDLRPGDTVTITCSSYQGYPEAEVFWQDGQGVPLTG NVTTSQMANEQGLFDVHSILRVVLGANGTYSCLVRNP VLQQDAHSSVTITPQRSPTGAVEVQVPEDPVVALVGT DATLRCSFSPEPGFSLAQLNLIWQLTDTKQLVHSFTEGR DQGSAYANRTALFPDLLAQGNASLRLQRVRVADEGSF TCFVSIRDFGSAAVSLQVAAPYSKPSMTLEPNKDLRPG DTVTITCSSYRGYPEAEVFWQDGQGVPLTGNVTTSQM ANEQGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAH GSVTITGQPMTFPPEALWVTVGLSVCLIALLVALAFVC WRKIKQSCEEENAGAEDQDGEGEGSKTALQPLKHSDS KEDDGQEIA
10	B7H4-IgV/B7H3-IgC	ORF	MASLGQILFWSIISIIIILAGAIALIIGFGISGRHSITVTTVAS AGNIGEDGILSCTFEPDIKLSDIVIQWLKEGVLGLVHEFK EGKDELSEQDEMFRGRTAVFADQVIVGNASLRLKNVQ LTDAGTYKCYIITSKGKGNANLEYKTGAPYSKPSMTLEP NKDLRPGDTVTITCSSYRGYPEAEVFWQDGQGVPLTG NVTTSQMANEQGLFDVHSVLRVVLGANGTYSCLVRNP VLQQDAHGSVTITGQPMTFPPEALWVTVGLSVCLIALL VALAFVCWRKIKQSCEEENAGAEDQDGEGEGSKTALQ PLKHSDSKEDDGQEIA
11	B7H3-IgV/B7H4-IgC	ORF	MLRRRGSPGMGVHVGAALGALWFCLTGALEVQVPED PVVALVGTDATLCCSFSPEPGFSLAQLNLIWQLTDTKQL VHSFAEGQDQGSAYANRTALFPDLLAQGNASLRLQRV RVADEGSFTCFVSIRDFGSAAVSLQVAAFSMPEVNVDY NASSETLRCEAPRWFPQPTVVWASQVDQGANFSEVS NTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIAK ATGDIKVTESEIKRRSHLQLLNSKASLCVSSFFAISWALLP LSPYLMLK

12	B7H4ECD- FcHisC	Mature protein	LIIGFGISGRHSITVTTVASAGNIGEDGILSCTFEPDIKLSD IVIQWLKEGVLGLVHEFKEGKDELSEQDEMFRGRTAVF ADQVIVGNASLRLKNVQLTDAGTYKCYIITSKGKGNAN LEYKTGAFSMPEVNVDYNASSETLRCEAPRWFPQPTV VWASQVDQGANFSEVSNTSFELNSENVTMKVVSVLY NVTINNTYSCMIENDIAKATGDIKVTESEIKRRSHLQLLN SKASIEGRMDPKSCDKTHTCPPCPAPEAEGAPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTAPP VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGKHHHHHHHHEPEA
13	Mature Human CD3ε (epsilon)	Mature protein	QDGNEEMGGITQTPYKVSISGTTVILTCPQYPGSEILW QHNDKNIGGDEDDKNIGSDEDHLSLKEFSELEQSGYYV CYPRGSKPEDANFYLYLRARVCENCMEMDVMSVATIV IVDICITGGLLLLVYYWSKNRKAKAKPVTRGAGAGGRQ RGQNKERPPPVPNPDYEPIRKGQRDLYSGLNQRRI
14	b12_VH	VH	QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWV RQAPGQRFEWMGWINPYNGNKEFSAKFQDRVTFTA DTSANTAYMELRSLRSADTAVYYCARVGPYSWDDSPQ DNYYMDVWGKGTTVIVSS
15	b12_VL	VL	EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQH KPGQAPRLVIHGVSNRASGISDRFSGSGSGTDFTLTITR VEPEDFALYYCQVYGASSYTFGQGTKLERK
16	VH_huCD3- H1L1	VH	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWV RQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRD DSKSSLYLQMNNLKTEDTAMYYCVRHGNFGNSYVSW FAYWGQGTLVTVSS
17	VH_huCD3- H1L1-H101G	VH	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWV RQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRD DSKSSLYLQMNNLKTEDTAMYYCVRGGNFGNSYVSW FAYWGQGTLVTVSS
18	VH_huCD3- H1L1_CDR1	VH_CDR1	GFTFNTYA
19	VH_huCD3- H1L1_CDR2	VH_CDR2	IRSKYNNYAT
20	VH_huCD3- H1L1_CDR3	VH_CDR3	VRHGNFGNSYVSWFAY
21	VH_huCD3- H1L1- H101G_CDR 3	VH_CDR3	VRGGNFGNSYVSWFAY
22	VL_huCD3- H1L1	VL	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANW VQQTPGQAFRGLIGGTNKRAPGVPARFSGSLIGDKAAL TITGAQADDESIYFCALWYSNLWVFGGGTKLTVL
23	VL_huCD3- H1L1_CDR1	VL_CDR1	TGAVTTSNY
	VL_huCD3- H1L1_CDR2	VL_CDR2	GTN

24	VL_huCD3-H1L1_CDR3	VL_CDR3	ALWYSNLWV
25	VH_B7H4-C1	VH	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYNPSLKSRVTISIDTSKNQFSLKLTSVTAADTAVFYCARGLFNWNFDSWGQGTLVTVSS
26	VH_B7H4-C1_CDR1	VH_CDR1	GGSFSGYY
27	VH_B7H4-C1_CDR2	VH_CDR2	INHSGST
28	VH_B7H4-C1_CDR3	VH_CDR3	ARGLFNWNFDS
29	VH_B7H4-C1-N52S	VH	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEISHSGSTNYNPSLKSRVTISIDTSKNQFSLKLTSVTAADTAVFYCARGLFNWNFDSWGQGTLVTVSS
30	VH_B7H4-C1-N52S _CDR2	VH_CDR2	ISHSGST
31	VH_B7H4-C1-N52Q	VH	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEIQHSGSTNYNPSLKSRVTISIDTSKNQFSLKLTSVTAADTAVFYCARGLFNWNFDSWGQGTLVTVSS
32	VH_B7H4-C1-N52Q_CDR2	VH_CDR2	IQHSGST
33	VL_B7H4-C1	VL	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYGASSLQSGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPRTFGQGTTVEIK
34	VL_B7H4-C1_CDR1	VL_CDR1	QGIRND
	VL_B7H4-C1_CDR2	VL_CDR2	GAS
35	VL_B7H4-C1_CDR3	VL_CDR3	LQHNSYPRT
36	VH_B7H4-C3	VH	EVQLVQSGAEVKKPGASVKVSCKASGYTFTNFWIHWVRQAPGQGLEWIGEIDPSDSYTNYNQKFKGRVTITRDTSTSTAYLELSSLRSEDTAVYYCAREITTVDYWGQGTLVTVSS
37	VH_B7H4-C3_CDR1	VH_CDR1	GYTFTNFW
38	VH_B7H4-C3_CDR2	VH_CDR2	IDPSDSYT
39	VH_B7H4-C3_CDR3	VH_CDR3	AREITTVDY
40	VL_B7H4-C3	VL	DIQMTQSPSSLSASVGDRVTITCSATSSISYMHWYQQKPGKAPKGWIYDTSKLAHGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCHQRRSYPFTFGQGTKVEIK
41	VL_B7H4-C3_CDR1	VL_CDR1	SSISY

	VL_B7H4-C3_CDR2	VL_CDR2	DTS
42	VL_B7H4-C3_CDR3	VL_CDR3	HQRRSYPFT
43	VH_B7H4-C2	VH	EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWIGWVRQAPGQGLEWIGDIYPGGGYTNYNEKFKGRVTITRDTSTSTAYLELSSLRSEDTAVYYCARLDGSSYRGAMDSWGQGTLVTVSS
44	VH_B7H4-C2_CDR1	VH_CDR1	GYTFTSYW
45	VH_B7H4-C2_CDR2	VH_CDR2	IYPGGGYT
46	VH_B7H4-C2_CDR3	VH_CDR3	ARLDGSSYRGAMDS
47	VL_B7H4-C2	VL	DIQMTQSPSSLSASVGDRVTITCKASQGFNKYVAWYQQKPGKAPKLLIYYTSTLQPGVPSRFSGSGSGRDYTLTISSLQPEDFATYYCLQYGNLLYAFGQGTKVEIK
48	VL_B7H4-C2_CDR1	VL_CDR1	QGFNKY
	VL_B7H4-C2_CDR2	VL_CDR2	YTS
49	VL_B7H4-C2_CDR3	VL_CDR3	LQYGNLLYA
50	VH_B7H4-C4	VH	EVQLVESGGGLIQPGGSLRLSCAASGFTVSSNYMNWVRQAPGKGLEWVSVIYGSGRTYYADSVKGRVTISRDNSKNTLYLQMNSLRAEDTAVYYCARDTYAMDVWGQGTTVTVSS
51	VH_B7H4-C4_CDR1	VH_CDR1	GFTVSSNY
52	VH_B7H4-C4_CDR2	VH_CDR2	IYGSGRT
53	VH_B7H4-C4_CDR3	VH_CDR3	ARDTYAMDV
54	VL_B7H4-C4	VL	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPMYTFGQGTKLEIK
55	VL_B7H4-C4_CDR1	VL_CDR1	QSVSSSY
	VL_B7H4-C4_CDR2	VL_CDR2	GAS
56	VL_B7H4-C4_CDR3	VL_CDR3	QQYGSSPMYT
57	IgG1-Fc	Constant	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

58	IgG1-Fc_F405L	Constant	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
59	IgG1-Fc_FEA	Constant	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
60	IgG1-Fc_FEAL	Constant	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
61	IgG1-Fc_FEAR	Constant	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
62	IgG1-Fc_K409R	Constant	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
63	Kappa	Constant	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
64	Lambda	Constant	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

65	VH_B7H4-C5	VH	QLQLQESGPGLVKPSETLSLTCTVSGGSIKSGSYYWGWIRQPPGKGLEWIGNIYYSGSTYYNPSLRSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAREGSYPNQFDPWGQGTLVTVSS
66	VH_B7H4-C5_CDR1	VH_CDR1	GGSIKSGSYY
67	VH_B7H4-C5_CDR2	VH_CDR2	IYYSGST
68	VH_B7H4-C5_CDR3	VH_CDR3	AREGSYPNQFDP
69	VL_B7H4-C5	VL	EIVMTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYHSFPFTFGGGTKVEIK
70	VL_B7H4-C5_CDR1	VL_CDR1	QSVSSN
	VL_B7H4-C5_CDR2	VL_CDR2	GAS
71	VL_B7H4-C5_CDR3	VL_CDR3	QQYHSFPFT

The CDR regions in the table listed above (CDR1, CDR2 and CDR3, and underlined sequences in VH and VL sequences) have been annotated according to IMGT (see Lefranc MP. et al., Nucleic Acids Research, 27, 209-212, 1999] and Brochet X. Nucl. Acids Res. 36, W503-508 (2008)). The references to K405L and K409R as used in the table above is in accordance with the Eu-index of numbering (described in Kabat, E.A. et al., Sequences of proteins of immunological interest. 5th Edition - US Department of Health and Human Services, NIH publication No. 91-3242, pp 662,680,689 (1991).

Detailed Description

Definitions

The term "antibody" as used herein is intended to refer to an immunoglobulin molecule, a fragment of an immunoglobulin molecule, or a derivative of either thereof, which has the ability to specifically bind to an antigen under typical physiological and/or tumor-specific conditions with a half-life of significant periods of time, such as at least about 30 minutes, at least about 45 minutes, at least about one hour, at least about two hours, at least about four hours, at least about 8 hours, at least about 12 hours, at least about 24 hours or more, at least about 48 hours or more, at least about 3, 4, 5, 6, 7 or more days, etc., or any other relevant functionally-defined period (such as a time sufficient to induce, promote, enhance, and/or modulate a physiological response associated with antibody binding to the antigen and/or time sufficient for the antibody to be internalized). An antibody comprises a binding

region (or binding domain which may be used herein, both having the same meaning) which can interact with an antigen, a binding region comprising variable regions of both heavy and light chains of an immunoglobulin molecule, or the like. Antibodies can comprise constant regions of the antibodies (Abs) which may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system such as C1q, the first component in the classical pathway of complement activation.

In the context of the present invention, the term “antibody” includes a monoclonal antibody (mAb), an antibody-like polypeptide, a chimeric antibody, a humanized antibody, as well as an ‘antibody fragment’ or a ‘fragment thereof’ retaining the ability to specifically bind to the antigen (antigen-binding fragment) provided by any known technique, such as enzymatic cleavage, peptide synthesis, and recombinant DNA technology. The term “antibody” includes bispecific antibodies and/or antibodies having further modifications, e.g. antibody-drug conjugates thereof.

An antibody as defined according to the invention can possess any isotype unless the disclosure herein is otherwise limited.

It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antibody" include (i) a Fab’ or Fab fragment, a monovalent fragment consisting of the light chain variable domain (VL), heavy chain variable domain (VH), light chain constant region (CL) and heavy chain constant region domain 1 (CH1) domains, or a monovalent antibody as described in WO 2007/059782; (ii) $F(ab')_2$ fragments, bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) an Fd fragment consisting essentially of the VH and CH1 domains; (iv) an Fv fragment consisting essentially of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment Ward et al., Nature 341, 544-546 (1989), which consists essentially of a VH domain and is also called domain antibody Holt et al; Trends Biotechnol. 2003 Nov;21(11):484-90; (vi) camelid or nanobodies Revets et al; Expert Opin Biol Ther. 2005 Jan;5(1):111-24 and (vii) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they may be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain antibodies or single chain Fv (scFv), see for instance Revets et al; Expert Opin Biol Ther. 2005 Jan;5(1):111-24 and Bird et al., Science 242, 423-426 (1988). Such single chain antibodies are encompassed within the term antibody unless otherwise noted or clearly indicated by context. Although such fragments are generally included within the meaning of antibody, they collectively and each independently are unique features of the present invention,

exhibiting different biological properties and utility. These and other useful antibody fragments in the context of the present invention are discussed further herein.

An antibody can be produced in and collected from different *in vitro* or *ex vivo* expression or production systems, for example from recombinantly modified host cells, from hybridomas or systems
5 that use cellular extracts supporting *in vitro* transcription and/or translation of nucleic acid sequences encoding the antibody. It is to be understood that a multitude of different antibodies, the antibodies being as defined in the context of the present invention, can be provided by producing each antibody separately in a production system as mentioned above and thereafter mixing the antibodies, or by producing several antibodies in the same production system.

10 The term “immunoglobulin heavy chain” or “heavy chain of an immunoglobulin” as used herein is intended to refer to one of the heavy chains of an immunoglobulin. A heavy chain is typically comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region (abbreviated herein as CH) which defines the isotype of the immunoglobulin. The heavy chain constant region typically is comprised of three domains, CH1, CH2, and CH3. The term
15 “immunoglobulin” as used herein is intended to refer to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains and one pair of heavy (H) chains, all four potentially inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized (see for instance Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Within the structure of the immunoglobulin, the two heavy
20 chains are inter-connected via disulfide bonds in the so-called “hinge region”. Equally to the heavy chains, each light chain is typically comprised of several regions; a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region typically is comprised of one domain, CL. Furthermore, the VH and VL regions may be further subdivided into regions of hypervariability (or hypervariable regions which may be hypervariable in sequence and/or
25 form of structurally defined loops), also termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

When used herein, the terms “half molecule”, “Fab-arm” and “arm” refer to one heavy chain-light
30 chain pair. When a bispecific antibody is described to comprise a half-molecule antibody “derived from” a first antibody, and a half-molecule antibody “derived from” a second antibody, the term “derived from” indicates that the bispecific antibody was generated by recombining, by any known method, said half-molecules from each of said first and second antibodies into the resulting bispecific antibody. In this context, “recombining” is not intended to be limited by any particular method of

recombining and thus includes all of the methods for producing bispecific antibodies described herein below, including for example recombining by half-molecule exchange, as well as recombining at nucleic acid level and/or through co-expression of two half-molecules in the same cells.

The term “antigen-binding region” or “binding region” as used herein, refers to a region of an antibody which is capable of binding to the antigen. The antigen can be any molecule, such as a polypeptide. Antigens may e.g. be presented on a cell, bacterium, or virion. The terms “antigen” and “target” may, unless contradicted by the context, be used interchangeably in the context of the present invention. The terms “antigen-binding region” and “antigen-binding site” may, unless contradicted by the context, be used interchangeably in the context of the present invention.

The term “blocks binding” or “blocking the binding of an antibody” or “cross-blocking binding” or “cross-blocks binding” refers to the situation where one antibody bound to a specific antigen prevents binding of the second antibody to the same antigen and *vice versa*. In the absence of the other antibody, each antibody has the ability to bind to the antigen as determined by a significant binding response, whereas one of the antibodies lacks a binding response when the other antibody is present. The ability of one antibody to block the binding of another antibody may be determined by biolayer interferometry in a classical sandwich epitope binning assay format, for instance as described in Example 5 in the present application and by Abdiche et al. (Abdiche YN, Malashock DS, Pinkerton A, Pons J. Exploring blocking assays using Octet, ProteOn, and Biacore biosensors. Anal Biochem. 2009; 386(2): 172-180). Briefly, in a sandwich epitope binning assay, an antibody in solution is tested for binding to its specific antigen that is first captured via an immobilized antibody. In the context of the present invention, one antibody does not block the binding of a second antibody if it is capable of binding to the antigen in the presence the second antibody and *vice versa*. The terms “blocks binding” and “blocking the binding of an antibody” and “cross-blocking binding” and “cross-blocks binding” may, unless contradicted by the context, be used interchangeably in the context of the present invention. An antibody that is said to blocks binding of another antibody, may also be said to compete with the other antibody for binding to the target.

The term “K_D” (M), as used herein, refers to the equilibrium dissociation constant of a particular antibody-antigen interaction, and is obtained by dividing k_d by k_a. K_D can also be referred to as “binding affinity”.

The term “k_d” (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{off} value or off-rate.

The term "k_a" (M^{-1} x sec^{-1}), as used herein, refers to the association rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{on} value or on-rate.

The term “binding” as used herein refers to the binding of an antibody to a predetermined antigen or target, typically with a binding affinity corresponding to a K_D of $1E^{-6}$ M or less, e.g. $5E^{-7}$ M or less, $1E^{-7}$ M or less, such as $5E^{-8}$ M or less, such as $1E^{-8}$ M or less, such as $5E^{-9}$ M or less, or such as $1E^{-9}$ M or less, when determined by biolayer interferometry using the antibody as the ligand and the antigen as the analyte and binds to the predetermined antigen with an affinity corresponding to a K_D that is at least ten-fold lower, such as at least 100-fold lower, for instance at least 1,000-fold lower, such as at least 10,000-fold lower, for instance at least 100,000-fold lower than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen.

The term “B7H4” as used herein, refers to a protein entitled B7H4, which is also referred to as: B7-H4; V-set domain containing T cell activation inhibitor 1; or VTCN1. B7H4 is a member of the B7 family of proteins, which family comprises cell-surface protein ligands that bind to receptors on lymphocytes. B7H4 is a type I transmembrane protein that includes a short intracellular domain, a hydrophobic transmembrane domain, and an extracellular domain with an IgV- and an IgC-like domain with four conserved cysteine residues and seven sites for N-linked glycosylation. (Sica et al., 2003, Immunity 18: 849-861). B7H4 proteins are known from various species, such as human (Homo sapiens) B7H4 (Uniprot accession no. Q7Z7D3), cynomolgus monkey (Macaca fascicularis) B7H4 transcript 1 (Uniprot accession no. A0A2K5U6P5), dog (Canis familiaris) B7H4 (Uniprot accession no. F1P8R9), rabbit (Oryctolagus cuniculus) B7H4 (Uniprot accession no. G1TQE8), rat (rattus norvegicus) B7H4 (Uniprot accession no. Q501W4), mouse (mus musculus) B7H4 (Uniprot accession no. Q7TSP5), and pig (sus scrofa) B7H4 (Uniprot accession no. F1SAY4). Natural variants of the listed B7H4 sequences may exist.

The term “CD3” as used herein, refers to the human Cluster of Differentiation 3 protein which is part of the T-cell co-receptor protein complex and is composed of four distinct chains. CD3 is found in various species, and thus, the term “CD3” may not be limited to human CD3, unless contradicted by context. In mammals, the complex contains a CD3γ (gamma) chain (human CD3γ chain UniProtKB/Swiss-Prot No P09693, or cynomolgus monkey CD3γ UniProtKB/Swiss-Prot No Q95LI7), a CD3δ (delta) chain (human CD3δ UniProtKB/Swiss-Prot No P04234, or cynomolgus monkey CD3δ UniProtKB/Swiss-Prot No Q95LI8), two CD3ε (epsilon) chains (human CD3ε: UniProtKB/Swiss-Prot No P07766, of which a sequence herein is incorporated as SEQ ID NO: 13, in which amino acid residues 1-22 represent a signal peptide and amino acid residues 23-207 represent the mature CD3ε polypeptide; cynomolgus monkey CD3ε UniProtKB/Swiss-Prot No Q95LI5; or rhesus monkey CD3ε UniProtKB/Swiss-Prot No G7NCB9), and a CD3ζ-chain (zeta) chain (human CD3ζ UniProtKB/Swiss-Prot No P20963, cynomolgus monkey CD3ζ UniProtKB/Swiss-Prot No Q09TK0). These chains associate with a molecule known as the T cell receptor (TCR) and generate an activation signal in T lymphocytes. The TCR and CD3 molecules together comprise the TCR complex.

The term “antibody binding region” refers to a region of the antigen, which comprises the epitope to which the antibody binds. An antibody binding region may be determined by epitope binning using biolayer interferometry, by alanine scan, or by domain shuffle assays (using antigen constructs in which regions of the antigen are exchanged with that of another species and determining whether the antibody still binds to the antigen or not). The amino acids within the antibody binding region that are involved in the interaction with the antibody may be determined by hydrogen/deuterium exchange mass spectrometry and/or by crystallography of the antibody bound to its antigen.

The term “epitope” means an antigenic determinant which is specifically bound by an antibody. Epitopes usually consist of surface groupings of molecules such as amino acids, sugar side chains or a combination thereof and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. The epitope may comprise amino acid residues which are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked or covered by the antibody when it is bound to the antigen (in other words, the amino acid residue is within or closely adjacent to the footprint of the specific antibody).

The terms "monoclonal antibody", “monoclonal Ab”, "monoclonal antibody composition", “mAb”, or the like, as used herein refer to a preparation of antibody molecules of single molecular composition and typically displays a single binding specificity and affinity for a particular epitope. A monoclonal antibody can be typically made by identical cells that are all clones of a unique parent cell, such as for example hybridomas, stable cell lines or the like. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. The human monoclonal antibodies may be produced by a hybridoma which includes a B cell obtained from a transgenic or transchromosomal nonhuman animal, such as a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene, fused to an immortalized cell. Human monoclonal antibodies may be derived from human B cells or plasma cells. Monoclonal antibodies may also be produced from recombinantly modified host cells, or systems that use cellular extracts supporting *in vitro* transcription and/or translation of nucleic acid sequences encoding the antibody.

The term “isotype” as used herein refers to the immunoglobulin class (for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) or any allotypes thereof, such as IgG1m(za) and IgG1m(f)) that is encoded by heavy chain constant region genes. Further, each heavy chain isotype can be combined with either a kappa (κ) or lambda (λ) light chain.

The term "full-length antibody" when used herein, refers to an antibody (e.g., a parent or variant antibody) comprising one pair of a heavy and light chain or two different pairs of heavy and light chains, each pair containing heavy and light chain constant and variable domains such as normally found in a heavy chain-light chain pair of a wild-type antibody of that isotype. In a full length variant antibody, the heavy and light chain constant and variable domains may in particular contain amino acid substitutions that modify and/or improve functional properties of the antibody when compared to the full length parent or wild-type antibody. A full-length antibody according to the present invention may be produced by a method comprising the steps of (i) cloning the CDR sequences into one or more suitable vectors comprising complete heavy and light chain sequences, and (ii) expressing the obtained suitable vectors with the heavy and light chain sequences in suitable expression systems. It is within the knowledge of the skilled person to produce a full-length antibody when starting out from either CDR sequences or full variable region sequences. Thus, the skilled person knows how to generate a full-length antibody in accordance with the present invention.

The term "humanized antibody" as used herein, refers to a genetically engineered non-human antibody, which contains human antibody constant domains and non-human variable domains modified to contain a high level of sequence homology to human variable domains. This can be achieved by grafting of non-human antibody complementarity-determining regions (CDRs), which together form the antigen binding site, onto a homologous human acceptor framework region (FR) (see i.a. WO92/22653 and EP0629240). In order to fully reconstitute the binding affinity and specificity of the parental antibody, substitution of framework residues from the parental antibody (i.e. the non-human antibody) into the human framework regions (back-mutations) may be required. Structural homology modeling may help to identify the amino acid residues in the framework regions that are important for the binding properties of the antibody. Thus, a humanized antibody may comprise non-human CDR sequences, primarily human framework regions optionally comprising one or more amino acid back-mutations to the non-human amino acid sequence, and fully human constant regions. Optionally, additional amino acid modifications, which are not necessarily back-mutations, may be applied to obtain a humanized antibody with preferred characteristics, such as particular useful affinity and biochemical properties, e.g. to include modifications to avoid deamidation, provide an "inert Fc region", and/or improve manufacturing.

The term "human antibody", as used herein, is intended to include antibodies having variable and framework regions derived from human germline immunoglobulin sequences and a constant domain derived from a human immunoglobulin constant domain. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations, insertions or deletions introduced by random or site-specific mutagenesis *in vitro* or by

somatic mutation *in vivo*). A "human antibody" can incorporate VH and VL sequences that have been generated from human germline immunoglobulin sequences in a human, in a transgenic animal such as described in the examples herein, a HIS mouse, or the like. Such VH and VL sequences are considered human VH and VL sequences, which have been e.g. fused to constant domains derived
5 from a human immunoglobulin constant domain.

Hence, "human antibodies" can be engineered antibodies. A "human antibody" may have been subjected to further engineering, e.g. include modifications to avoid deamidation, provide an "inert Fc region", enable bispecific antibody generation and/or improve manufacturing. A human antibody may also be produced in non-human cells, e.g. in CHO cells or the like. However, the term "human
10 antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another non-human species, such as a mouse, have been grafted onto human framework sequences.

The term "Fc region" as used herein, refers to a region comprising, in the direction from the N- to C-terminal ends of the two heavy chains of the antibody, at least a hinge region, a CH2 region and a CH3
15 region. An Fc region of the antibody may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system.

The term "hinge region" as used herein refers to the hinge region of an immunoglobulin heavy chain. Thus, for example the hinge region of a human IgG1 antibody corresponds to amino acids 216-230
20 according to the Eu numbering as set forth in Kabat Kabat, E.A. et al., Sequences of proteins of immunological interest. 5th Edition - US Department of Health and Human Services, NIH publication No. 91-3242, pp 662,680,689 (1991). However, the hinge region may also be any of the other subtypes as described herein.

The term "CH1 region" or "CH1 domain" as used herein refers to the CH1 region of an immunoglobulin
25 heavy chain. Thus, for example the CH1 region of a human IgG1 antibody corresponds to amino acids 118-215 according to the Eu numbering as set forth in Kabat (*ibid*). However, the CH1 region may also be any of the other subtypes as described herein.

The term "CH2 region" or "CH2 domain" as used herein refers to the CH2 region of an immunoglobulin heavy chain. Thus, for example the CH2 region of a human IgG1 antibody corresponds to amino acids
30 231-340 according to the Eu numbering as set forth in Kabat (*ibid*). However, the CH2 region may also be any of the other subtypes as described herein.

The term "CH3 region" or "CH3 domain" as used herein refers to the CH3 region of an immunoglobulin heavy chain. Thus, for example the CH3 region of a human IgG1 antibody corresponds to amino acids

341-447 according to the Eu numbering as set forth in Kabat (*ibid*). However, the CH3 region may also be any of the other subtypes as described herein.

The term "Fc-mediated effector functions," as used herein, is intended to refer to functions that are a consequence of binding a polypeptide or antibody to its target or antigen on a cell membrane wherein
5 the Fc-mediated effector function is attributable to the Fc region of the polypeptide or antibody. Examples of Fc-mediated effector functions include (i) C1q binding, (ii) complement activation, (iii) complement-dependent cytotoxicity (CDC), (iv) antibody-dependent cell-mediated cytotoxity (ADCC), (v) Fc-gamma receptor (FcgR)-binding, (vi) antibody-dependent, FcγR-mediated antigen crosslinking, (vii) antibody-dependent cellular phagocytosis (ADCP), (viii) complement-dependent cellular
10 cytotoxicity (CDCC), (ix) complement-enhanced cytotoxicity, (x) binding to complement receptor of an opsonized antibody mediated by the antibody, (xi) opsonisation, and (xii) a combination of any of (i) to (xi).

The term "inertness", "inert" or "non-activating" as used herein, refers to an Fc region which is at least not able to bind any FcγR, induce Fc-mediated cross-linking of FcγRs, or induce FcγR-mediated cross-
15 linking of target antigens via two Fc regions of individual antibodies, or is not able to bind C1q. An example thereof is FEA substitutions within the constant domain as described herein. The inertness of an Fc region of an antibody, may be tested using the antibody in a monospecific or bispecific format.

The term "full-length" when used in the context of an antibody indicates that the antibody is not a fragment, but contains all of the domains corresponding with the particular isotype such as normally
20 found for that isotype in nature, e.g. the VH, CH1, CH2, CH3, hinge, VL and CL domains for an IgG1 antibody.

The term "monovalent antibody", in the context of the present invention, refers to an antibody molecule that can interact with an antigen, with only one antigen-binding domain (e.g. one Fab arm). In the context of a bispecific antibody, "monovalent antibody binding" refers to the binding of the
25 bispecific antibody to one antigen with only one antigen-binding domain (e.g. one Fab arm).

The term "monospecific antibody" in the context of the present invention, refers to an antibody that has binding specificity to one antigen, one epitope only. The antibody may be a monospecific, monovalent antibody (i.e. carrying only one antigen-binding region) or a monospecific, bivalent antibody (e.g. an antibody with two identical antigen-binding regions).

30 The term "bispecific antibody" refers to an antibody having two antigen-binding domains that bind different epitopes, e.g. two non-identical pairs of VH and VL regions, two non-identical Fab-arms or two Fab-arms with non-identical CDR regions. In the context of this invention, bispecific antibodies

have specificity for at least two different epitopes. Such epitopes may be on the same or different antigens or targets. If the epitopes are on different antigens, such antigens may be on the same cell or different cells, cell types or structures, such as extracellular matrix or vesicles and soluble protein. A bispecific antibody may thus be capable of crosslinking multiple antigens, e.g. two different cells.

5 The term “bivalent antibody” refers to an antibody that has two antigen-binding regions, which bind to two of the same epitopes on two of the same antigens or binds to two different epitopes on the same or different antigen(s). Hence, a bivalent antibody may be a monospecific antibody or a bispecific antibody.

The term “amino acid” and “amino acid residue” may herein be used interchangeably, and are not to 10 be understood limiting. Amino acids are organic compounds containing amine (-NH_2) and carboxyl (-COOH) functional groups, along with a side chain (R group) specific to each amino acid. In the context of the present invention, amino acids may be classified based on structure and chemical characteristics. Thus, classes of amino acids may be reflected in one or both of the following tables:

Main classification based on structure and general chemical characterization of R group

15

TABLE 2

Class	Amino acid
Acidic Residues	D and E
Basic Residues	K, R, and H
Hydrophilic Uncharged Residues	S, T, N, and Q
Aliphatic Uncharged Residues	G, A, V, L, and I
Non-polar Uncharged Residues	C, M, and P
Aromatic Residues	F, Y, and W

TABLE 3

Alternative Physical and Functional Classifications of Amino Acid Residues

Class	Amino acid
Hydroxyl group containing residues	S and T
Aliphatic residues	I, L, V, and M
Cycloalkenyl-associated residues	F, H, W, and Y
Hydrophobic residues	A, C, F, G, H, I, L, M, R, T, V, W, and Y
Negatively charged residues	D and E

Polar residues	C, D, E, H, K, N, Q, R, S, and T
Positively charged residues	H, K, and R
Small residues	A, C, D, G, N, P, S, T, and V
Very small residues	A, G, and S
Residues involved in turn formation	A, C, D, E, G, H, K, N, Q, R, S, P, and T
Flexible residues	Q, T, K, S, G, P, D, E, and R

Substitution of one amino acid for another may be classified as a conservative or non-conservative substitution. In the context of the invention, a “conservative substitution” is a substitution of one amino acid with another amino acid having similar structural and/or chemical characteristics, such
5 substitution of one amino acid residue for another amino acid residue of the same class as defined in any of the two tables above: for example, leucine may be substituted with isoleucine as they are both aliphatic, branched hydrophobes. Similarly, aspartic acid may be substituted with glutamic acid since they are both small, negatively charged residues.

10 In the context of the present invention, a substitution in an antibody is indicated as:
Original amino acid – position – substituted amino acid;
Referring to the well-recognized nomenclature for amino acids, the three letter code, or one letter code, is used, including the codes “Xaa” or “X” to indicate any amino acid residue. Thus, Xaa or X may typically represent any of the 20 naturally occurring amino acids. The term “naturally occurring” as
15 used herein refers to any one of the following amino acid residues; glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, arginine, histidine, aspartic acid, asparagine, glutamic acid, glutamine, proline, tryptophan, phenylalanine, tyrosine, methionine, and cysteine.

Accordingly, the notation “K409R” or “Lys409Arg” means, that the antibody comprises a substitution
20 of Lysine with Arginine in amino acid position 409. Substitution of an amino acid at a given position to any other amino acid is referred to as: Original amino acid – position; or e.g. “K409”. For a modification where the original amino acid(s) and/or substituted amino acid(s) may comprise more than one, but not all amino acid(s), the more than one amino acid may be separated by “,” or “/”. E.g. the substitution of Lysine with Arginine, Alanine, or Phenylalanine in position 409 is: “Lys409Arg,Ala,Phe”
25 or “Lys409Arg/Ala/Phe” or “K409R,A,F” or “K409R/A/F” or “K409 to R, A, or F”. Such designation may be used interchangeably in the context of the invention but have the same meaning and purpose.

Furthermore, the term “a substitution” embraces a substitution into any one or the other nineteen

natural amino acids, or into other amino acids, such as non-natural amino acids. For example, a substitution of amino acid K in position 409 includes each of the following substitutions: 409A, 409C, 409D, 409E, 409F, 409G, 409H, 409I, 409L, 409M, 409N, 409Q, 409R, 409S, 409T, 409V, 409W, 409P, and 409Y. This is, by the way, equivalent to the designation 409X, wherein the X designates any amino
5 acid other than the original amino acid. These substitutions may also be designated K409A, K409C, etc. or K409A,C, etc. or K409A/C/etc. The same applies by analogy to each and every position mentioned herein, to specifically include herein any one of such substitutions.

The antibody according to the invention may also comprise a deletion of an amino acid residue. Such deletion may be denoted “del”, and includes, e.g., writing as K409del. Thus, in case of such
10 embodiments, the Lysine in position 409 has been deleted from the amino acid sequence.

The term "host cell", as used herein, is intended to refer to a cell into which a nucleic acid such as an expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell, but may also include the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental
15 influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. Recombinant host cells include, for example, transfectomas, such as CHO cells, HEK-293 cells, Expi293F cells, PER.C6 cells, NS0 cells, and lymphocytic cells, and prokaryotic cells such as *E. coli* and other eukaryotic hosts such as plant cells and fungi.

20 The term “transfectoma”, as used herein, includes recombinant eukaryotic host cells expressing the antibody or a target antigen, such as CHO cells, PER.C6 cells, NS0 cells, HEK-293 cells, Expi293F cells, plant cells, or fungi, including yeast cells.

For purposes of the present invention, sequence identity between two amino acid sequences is determined over the length of the referenced sequence using the Needleman-Wunsch algorithm
25 (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief
30 option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment - Total Number of Gaps in Alignment).

The retention of similar residues may also or alternatively be measured by a similarity score, as determined by use of a BLAST program (e.g., BLAST 2.2.8 available through the NCBI using standard

settings BLOSUM62, Open Gap=11 and Extended Gap=1). Suitable variants typically exhibit at least about 45%, such as at least about 55%, at least about 65%, at least about 75%, at least about 85%, at least about 90%, at least about 95%, or more (e.g., about 99%) similarity to the parent or referenced sequence.

5 The term “internalized” or “internalization” as used herein, refers to a biological process in which molecules such as the antibody according to the present invention, are engulfed by the cell membrane and drawn into the interior of the cell. Internalization may also be referred to as “endocytosis”.

Pharmaceutical compositions and unit dosage forms comprising bispecific antibodies targeting CD3xB7H4

10 In a first aspect of the invention, a pharmaceutical composition or a unit dosage form is provided comprising an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said antigen-binding regions are human variable regions and/or humanized variable regions. For example, one antigen-binding region may
15 comprise human heavy and light chain variable regions, and the other antigen-binding region may comprise humanized heavy and light chain variable regions. Or, both antigen-binding region may comprise human heavy and light chain variable regions, or both antigen-binding regions may comprise humanized heavy and light chain variable regions. Hence, accordingly, a pharmaceutical composition or a unit dosage form is provided comprising an antibody comprising an antigen-binding region
20 capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions comprise human framework regions. An antibody in accordance with the invention as described herein comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, may also be referred
25 to herein e.g. as a CD3xB7H4 antibody.

Such antibodies are preferably bispecific antibodies. Such an antibody as described above are in a further embodiment capable of binding cancer cells and T-cells, such as e.g. described in the examples. Cancer cells that may be selected are cancer cells that express human B7H4 and/or are cancer cells that are of a solid tumor. Such an antibody preferably is capable of inducing T-cell mediated cell killing
30 of the cancer cells.

Capable of binding is understood to comprise, as shown in the examples, that in a binding assay, an antibody binds to its target, as shown by e.g. typical binding curves such as shown in Figures 3 and 4 herein, or by determining binding affinity, using e.g. biolayer interferometry, as shown in examples 3

and 4. An antigen-binding region not capable of binding to a specified target has e.g. an undectable binding affinity to its target, e.g. having a response of < 0.05 nm at the highest concentration used in a typical biolayer interferometry assay such as shown in example 3. In any case, the skilled person is well aware how to determine whether or not an antigen-bindig region is capable of binding to its target.

In a first aspect, the present invention relates to a pharmaceutical composition comprising:

a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions are humanized and/or human, and

b) a buffering agent,

wherein the pH of the composition is from 4.0 to 8.0.

It is preferred that the pH is from 4.5 to 6.5. In a preferred embodiment, the pH of the composition is from 5.0 to 6.5. In a particularly preferred embodiment, the the pH of the composition is from 5.0 to 6.0. It has been found that these pH ranges result in a particularly high stability of the antibody, in particular thermal stability. Also, at the pH range of the present invention the pharmaceutical compositions provide a high degree of solubility of the antibody.

In a preferred embodiment, the buffering agent is selected from the group consisting of histidine, glutamate, and mixtures thereof.

In a preferred embodiment, the pharmaceutical composition further comprises c) a non-ionic excipient.

In a preferred embodiment, the non-ionic excipient is a sugar or a sugar alcohol.

In anembodiment, the non-ionic excipient is selected from sorbitol, sucrose or mixtures thereof. In a preferred embodiment, the non-ionic excipient is sorbitol.

In a preferred embodiment, the non-ionic excipient is present at a concentration of 100 to 300 mM, such as 125-250 mM preferably 250 mM. In a preferred embodiment, the non-ionic excipient is sorbitol and it is present at a concentration of between 125-250 mM, preferably 250 mM.

In a preferred embodiment, the pharmaceutical composition further comprises d) a surfactant.

In some embodiments, the surfactant is selected from the group consisting of glycerol monooleate, benzethonium chloride, sodium docusate, phospholipids, polyethylene alkyl ethers, sodium lauryl sulfate and tricaprylin, benzalkonium chloride, citrimide, cetylpyridinium chloride and phospholipids,

alpha tocopherol, glycerol monooleate, myristyl alcohol, phospholipids, poloxamers, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbintan fatty acid esters, polyoxyethylene sterarates, polyoxyl hydroxystearate, polyoxylglycerides, polysorbates, propylene glycol dilaurate, propylene glycol monolaurate, sorbitan esters sucrose palmitate, sucrose stearate, tricaprylin and TPGS, and mixtures thereof.

In an embodiment, the surfactant is a polysorbate, preferably polysorbate 20 or 80. In a preferred embodiment the surfactant is polysorbate 80.

In a preferred embodiment, the surfactant is present at a concentration from about 0.005% to 0.4% w/v, such as from about 0.01 to 0.1 % w/v, such as from about 0.01 to 0.09 % w/v such as from about 0.01 to 0.06 % w/v such as from about 0.01 to 0.05% w/v such as 0.02% w/v or 0.03% w/v or 0.04% w/v or 0.05% w/v, preferably 0.02% w/v. In a preferred embodiment the surfactant is polysorbate 80 and it is present at between 0.02% w/v to 0.04 w/v in the pharmaceutical formulation. Preferably it is 0.02% w/v.

In a preferred embodiment, the concentration of the antibody is 0.5 to 100 mg/ml, such as 1.0 to 50 mg/ml, or such as 5 to 30 mg/ml, such as 5 mg/ml, or 6 mg/ml, or 7 mg/ml, or 8 mg/ml, or 9 mg/ml, or 10 mg/ml, or 11 mg/ml, or 12 mg/ml, or 13 mg/ml, or 14 mg/ml, or 15 mg/ml, or 16 mg/ml, or 17 mg/ml, or 18 mg/ml, or 19 mg/ml, or 20 mg/ml, or 21 mg/ml, or 22 mg/ml, or 23 mg/ml, or 24 mg/ml, or 25 mg/ml, or 26 mg/ml, or 27 mg/ml, or 28 mg/ml, or 29 mg/ml, 30 mg/ml, 31 mg/ml, 32 mg/ml, 33 mg/ml, 34 mg/ml, 35 mg/ml, 36 mg/ml, 37 mg/ml, 38 mg/ml, 39 mg/ml, 40 mg/ml, 41 mg/ml, 42 mg/ml, 43 mg/ml, 44 mg/ml, 45 mg/ml, 46 mg/ml, 47 mg/ml, 48 mg/ml, 49 mg/ml, 50 mg/ml, 51 mg/ml, 52 mg/ml, 53 mg/ml, 54 mg/ml, 55 mg/ml, 56 mg/ml, 57 mg/ml, 58 mg/ml, 59 mg/ml, or such as 60 mg/ml. Most preferred the concentration is between 10 to 20 mg/ml such as 20 mg/ml. However, it may also be present at 10 mg/ml. In another embodiment it is present at 15 mg/ml. In another embodiment it is present at 25 mg/ml. In another embodiment it is present at 30 mg/ml. In another embodiment it is present at 35 mg/ml. In another embodiment it is present at 40 mg/ml. In another embodiment it is present at 45 mg/ml. In another embodiment it is present at 50 mg/ml. In another embodiment it is present at 55 mg/ml. In another embodiment it is present at 60 mg/ml.

In a preferred embodiment, the buffering agent is present at a concentration of 5 to 40 mM, such as 10-30 mM, preferably 20 mM. In a preferred embodiment the buffer is 20 mM glutamate at pH 5.1 to 5.3, such as 5.2. In yet another preferred embodiment the buffer is 20 mM histidine at pH 5.7 to 5.9, such as 5.8.

In a preferred embodiment, the pharmaceutical composition is a liquid composition. In a preferred

embodiment, the pharmaceutical composition is an aqueous composition.

In a preferred embodiment, the pharmaceutical composition comprises:

a) 5-50 mg/ml of the antibody, preferably 5-25 mg/ml of the antibody, most preferably 10-20 mg/ml of the antibody

5 b) 10 to 20 mM glutamate, preferably 15-20 mM glutamate, most preferably 20 mM glutamate

c) 150 to 350 mM sorbitol, preferably 200-300 mM sorbitol, most preferably 250 mM sorbitol,

d) a polysorbate, preferably polysorbate 80, most preferably 0.02% w/v polysorbate 80,

wherein the pH of the composition is from 5.0 to 6.0, preferably 5.1-5.3.

In a preferred embodiment, the pharmaceutical composition is:

10 a pharmaceutical composition comprising a) 10-20 mg/ml of the antibody, b) 20 mM glutamate, c) 250 mM sorbitol, d) 0.02% w/v polysorbate 80, wherein the pH of the composition is from 5.1-5.3.

In another preferred embodiment the pharmaceutical composition is:

a pharmaceutical composition comprising a) 10-20 mg/ml of the antibody, b) 20 mM histidine, c)
15 250 mM sorbitol, d) 0.02% w/v polysorbate 80, wherein the pH of the composition is from 5.7-5.9.

In another embodiment, the composition is an intravenous composition, and/or wherein the composition is for use in intravenous administration.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-
20 binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises a heavy chain variable region (VH), wherein CDR1 is according to SEQ ID NO. 18, wherein CDR2 is according to SEQ ID NO. 19, and wherein CDR3 is according to SEQ ID NO. 21.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-
25 binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 23, wherein CDR2 is GTN, and wherein CDR3 is according to SEQ ID NO: 24.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-
30 binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to

human CD3, wherein the antigen-binding region capable of binding to B7H4 comprises a variable heavy chain (VH) region, wherein CDR1 is according to SEQ ID NO. 26, wherein CDR2 is according to SEQ ID NO. 30, and wherein CDR3 is according to SEQ ID NO. 28.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-
5 binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region capable of binding to B7H4 comprises a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 34, wherein CDR2 is GAS, and wherein CDR3 is according to SEQ ID NO: 35.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-
10 binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH), wherein ÇDR1 is according to SEQ ID NO. 18, wherein CDR2 is according to SEQ ID NO. 19, and wherein CDR3 is according to SEQ ID NO. 21, and

a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 23, wherein CDR2
15 is GTN, and wherein CDR3 is according to SEQ ID NO: 24, and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region, wherein ÇDR1 is according to SEQ ID NO. 26, wherein CDR2 is according to SEQ ID NO. 30, and wherein CDR3 is according to SEQ ID NO. 28, and

a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 34, wherein CDR2
20 is GAS, and wherein CDR3 is according to SEQ ID NO: 35.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID
25 NO. 17, and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22, and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain (VL) region comprising the CDR1, CDR2 and CDR3 regions
30 respectively of SEQ ID NO. 33, wherein the CDR regions may be numbered according to IMGT.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to

human CD3 wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO. 17, and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 22, and

5 wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the amino acid sequence of SEQ ID NO. 29, and a variable light chain (VL) region comprising the amino acid sequence of SEQ ID NO. 33.

In a preferred embodiment, the antibody of the pharmaceutical formulation comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to
10 human CD3 wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO. 17, and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 22, and further comprises a constant heavy chain region (CH) comprising the the amino acid sequence of SEQ ID NO. 60 and a constant light chain region (CL) comprising the the amino
15 acid sequence of SEQ ID NO. 64, and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the amino acid sequence of SEQ ID NO. 29, and a variable light chain (VL) region comprising the amino acid sequence of SEQ ID NO. 33, and further comprises a constant heavy chain region (CH) comprising the the amino acid
20 sequence of SEQ ID NO. 61 and a constant light chain region (CL) comprising the the amino acid sequence of SEQ ID NO. 63.

In a particularly preferred embodiment, the antibody of the pharmaceutical formulation is bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, or a biosimilar thereof.

The present invention also relates to the pharmaceutical composition as defined herein for use as a
25 medicament, such as for use in a method for the treatment of a disease. In a preferred embodiment, the disease is cancer. In a preferred embodiment, the cancer is characterized by expression of B7H4 in cancer cells. Preferably, said expression of B7H4 is determined in cancer cells obtained from a patient.

In a preferred embodiment, the cancer is a solid tumor. Preferably, the cancer is selected from the
30 group consisting of lung cancer, NSCLC (ADC or SQCC), stomach cancer, pancreas cancer, cholangiocarcinoma, bladder cancer, cervical cancer, head and neck cancer, breast cancer, ovarian cancer and uterine cancer.

In another aspect, the present invention relates to a method of treating a disease, the method comprising administering a pharmaceutical composition as defined herein to a subject in need thereof. In a preferred embodiment, said method is for treatment of a cancer. In some embodiments, the cancer is selected from the group consisting of uterine carcinosarcoma (UCS), bladder urothelial carcinoma (BLCA), pancreatic adenocarcinoma (PAAD), lung squamous cell carcinoma (LUSC), breast invasive carcinoma (BRCA), uterine corpus endometrial carcinoma (UCEC), ovarian serous cystadenocarcinoma (OV) and cholangiocarcinoma (CHOL).

In another aspect, the present invention relates to a method of treating cancer in a subject comprising administering to a subject in need thereof the pharmaceutical composition of the present invention for a time sufficient to treat the cancer. In some embodiments, the composition is administered intravenously.

In another aspect, the present invention relates to a use of the pharmaceutical composition of the present invention for intravenous administration. In a preferred embodiment, the use is for the treatment of cancer.

In another aspect, the present invention relates to a unit dosage form, comprising

a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions are humanized and/or human, in an amount of from 5 pg to 120 mg, and

b) a buffering agent, preferably selected from the group consisting of histidine, glutamate, and mixtures thereof,

wherein the pH of the unit dosage form is from 4.0 to 8.0, preferably 4.5 to 6.5, more preferably 5.0 to 6.0.

Preferably, the amount of the antibody is from 40 pg to 8 g. In some embodiments, the amount of the antibody is from 40 pg to 60 mg, such as 40 pg, 50 pg, 100 pg, 150, 160 pg, 170 pg, 180 pg, 190 pg, 200 pg, 250 pg, 300 pg, 350 pg, 400 pg, 450 pg, 500 pg, 600 pg, 700 pg, 800 pg, 900 pg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg, 16 mg, 17 mg, 18 mg, 19 mg, 20 mg, 21 mg, 22 mg, 23 mg, 24 mg, 25 mg, 26 mg, 27 mg, 28 mg, 29 mg, 30 mg, 31 mg, 32 mg, 33 mg, 34 mg, 35 mg, 36 mg, 37 mg, 38 mg, 39 mg, 40 mg, 41 mg, 42 mg, 43 mg, 44 mg, 45 mg, 46 mg, 47 mg, 48 mg, 49 mg, 50 mg, 51 mg, 52 mg, 53 mg, 54 mg, 55 mg, 56 mg, 57 mg, 58 mg, 59 mg, 60 mg, 65 mg, 70mg, 75 mg, 80 mg, 90 mg, 100 mg, 110 mg, 120mg, 130 mg, 140mg, 150 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 550 mg, 600 mg, 650 mg, 700 mg, 750 mg, 800

mg, 850 mg, 900 mg, 950 mg, 1000 mg, 1050 mg, 1100 mg, 1150 mg, 1200 mg, 1250 mg, 1300 mg, 1350 mg, 1400 mg, 1500 mg, 2000 mg, 3000 mg, 4000 mg, 5000 mg, 6000 mg, or such as 7000 mg.

In another preferred embodiment, the total volume of the unit dosage form is from 20 ml to 200 ml, wherein the dosage form is for I.V. administration.

5 In a preferred embodiment, the unit dosage form comprises a non-ionic excipient, preferably a sugar or a sugar alcohol. Preferably, the non-ionic excipient is selected from sorbitol, sucrose or mixtures thereof. In an advantageous embodiment, the non-ionic excipient is present at a concentration of 100 to 300 mM, such as 125-250 mM preferably 250 mM.

It is preferred that the unit dosage form further comprises d) a surfactant. In some embodiments, the 10 surfactant is selected from the group consisting of glycerol monooleate, benzethonium chloride, sodium docusate, phospholipids, polyethylene alkyl ethers, sodium lauryl sulfate and tricaprylin, benzalkonium chloride, citrimide, cetylpyridinium chloride and phospholipids, alpha tocopherol, glycerol monooleate, myristyl alcohol, phospholipids, poloxamers, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbintan fatty acid esters, polyoxyethylene 15 sterarates, polyoxyl hydroxystearate, polyoxylglycerides, polysorbates, propylene glycol dilaurate, propylene glycol monolaurate, sorbitan esters sucrose palmitate, sucrose stearate, tricaprylin and TPGS, and mixtures thereof. In a preferred embodiment, the surfactant is a polysorbate, preferably polysorbate 20 or 80, most preferably polysorbate 80. In a preferred embodiment, the surfactant is present at a concentration from about 0.005% to 0.4% w/v, such as from about 0.01 to 0.1 % w/v, 20 such as from about 0.01 to 0.09 % w/v such as from about 0.01 to 0.06 % w/v such as from about 0.01 to 0.05% w/v such as 0.02% w/v or 0.03% w/v or 0.04% w/v or 0.05% w/v, preferably 0.02% w/v.

In a preferred embodiment, the concentration of the antibody in the unit dosage form is 0.5 to 100 mg/ml, such as 1.0 to 50 mg/ml, or such as 5 to 30 mg/ml, such as 5 mg/ml, or 6 mg/ml, or 7 mg/ml, or 8 mg/ml, or 9 mg/ml, or 10 mg/ml, or 11 mg/ml, or 12 mg/ml, or 13 mg/ml, or 14 mg/ml, or 15 25 mg/ml, or 16 mg/ml, or 17 mg/ml, or 18 mg/ml, or 19 mg/ml, or 20 mg/ml, or 21 mg/ml, or 22 mg/ml, or 23 mg/ml, or 24 mg/ml, or 25 mg/ml, or 26 mg/ml, or 27 mg/ml, or 28 mg/ml, or 29 mg/ml, 30 mg/ml, 31 mg/ml, 32 mg/ml, 33 mg/ml, 34 mg/ml, 35 mg/ml, 36 mg/ml, 37 mg/ml, 38 mg/ml, 39 mg/ml, 40 mg/ml, 41 mg/ml, 42 mg/ml, 43 mg/ml, 44 mg/ml, 45 mg/ml, 46 mg/ml, 47 mg/ml, 48 mg/ml, 49 mg/ml, 50 mg/ml, 51 mg/ml, 52 mg/ml, 53 mg/ml, 54 mg/ml, 55 mg/ml, 56 mg/ml, 57 30 mg/ml, 58 mg/ml, 59 mg/ml, or such as 60 mg/ml.

In a preferred embodiment, the buffering agent is present in the unit dosage form at a concentration of 5 to 40 mM, such as 10-30 mM, preferably 20 mM.

In a preferred embodiment, the unit dosage form is a liquid unit dosage form.

In another aspect, the present invention relates to a method of treating cancer in a subject comprising administering to a subject in need thereof the unit dosage form of the present invention for a time sufficient to treat the cancer. In another aspect, the present invention relates to a unit dosage form of the present invention for use in the treatment of cancer.

5 In yet another aspect, the present invention relates to a container comprising the unit dosage form of the present invention or the pharmaceutical composition of the present invention. The container may be made of glass or one or more polymer materials.

In another embodiment, the present invention relates to a kit-of-parts comprising:

a. the pharmaceutical composition of the present invention, or the unit dosage form of any of the
10 present invention, b. a receptacle for the pharmaceutical composition or for the unit dosage form, and c. directions for dilution and/or for use.

In another aspect, the present invention relates to a kit-of-parts comprising:

a. the pharmaceutical composition of the present invention, or the unit dosage form of any of the present invention, b. a diluent, c. a receptacle for the unit dosage form, and d. directions for dilution
15 and/or for use.

In another aspect, the present invention relates to a kit-of-parts, such as a kit for use as a companion diagnostic/for identifying within a population of patients those patients which have a propensity to respond to treatment with a pharmaceutical composition as defined herein, comprising a pharmaceutical composition as defined herein; and instructions for use of said kit.

20 In another aspect, the present invention relates to a method of preparing a pharmaceutical composition as defined herein, comprising the steps of mixing in water for injection: a. 0.5 to 120 mg/ml of the antibody, and b. a buffering agent and adjusting the pH to 4.0-8.0, preferably to 5.0-6.0.

In another aspect, the present invention relates to a method of preparing a unit dosage form as defined herein, comprising the steps of:

25 a. preparing the pharmaceutical composition by the steps of the above-described method of preparing a pharmaceutical composition, or providing a pharmaceutical composition as defined herein; b. providing a diluent, and c. mixing the pharmaceutical composition and the diluent to a desired antibody concentration.

In another aspect, the present invention relates to a pharmaceutical composition or a unit dosage
30 form, which is obtainable by any of the afore-described methods.

Bispecific formats

The present invention provides pharmaceutical compositions or unit dosage forms comprising bispecific CD3xB7H4 antibodies which efficiently promote T cell-mediated killing of B7H4-expressing tumor cells. Depending on the desired functional properties for a particular use, particular antigen-binding regions can be selected from the set of antibodies or antigen-binding regions provided by the present invention. Many different formats and uses of bispecific antibodies are known in the art, and were reviewed by Kontermann; Drug Discov Today, 2015 Jul;20(7):838-47 and; MAbs, 2012 Mar-Apr;4(2):182-97. A bispecific antibody according to the present invention may not be limited to any particular bispecific format or method of producing it.

Examples of bispecific antibody molecules which may be used in the present invention comprise (i) a single antibody that has two arms comprising different antigen-binding regions; (ii) a single chain antibody that has specificity to two different epitopes, e.g., via two scFvs linked in tandem by an extra peptide linker; (iii) a dual-variable-domain antibody (DVD-Ig), where each light chain and heavy chain contains two variable domains in tandem through a short peptide linkage (Wu et al., Generation and Characterization of a Dual Variable Domain Immunoglobulin (DVD-Ig™) Molecule, In: Antibody Engineering, Springer Berlin Heidelberg (2010)); (iv) a chemically-linked bispecific (Fab')2 fragment; (v) a Tandab, which is a fusion of two single chain diabodies resulting in a tetravalent bispecific antibody that has two binding sites for each of the target antigens; (vi) a flexibody, which is a combination of scFvs with a diabody resulting in a multivalent molecule; (vii) a so-called "dock and lock" molecule, based on the "dimerization and docking domain" in Protein Kinase A, which, when applied to Fabs, can yield a trivalent bispecific binding protein consisting of two identical Fab fragments linked to a different Fab fragment; (viii) a so-called Scorpion molecule, comprising, e.g., two scFvs fused to both termini of a human Fab-arm; and (ix) a diabody.

In one embodiment, the bispecific antibody of the present invention is a diabody, a cross-body, or a bispecific antibody obtained via a controlled Fab-arm exchange also know as DuoBody® (such as described in WO2011131746 (Genmab)).

Examples of different classes of bispecific antibodies include but are not limited to (i) IgG-like molecules with complementary CH3 domains to force heterodimerization; (ii) recombinant IgG-like dual targeting molecules, wherein the two sides of the molecule each contain the Fab fragment or part of the Fab fragment of at least two different antibodies; (iii) IgG fusion molecules, wherein full length IgG antibodies are fused to extra Fab fragment or parts of Fab fragment; (iv) Fc fusion molecules, wherein single chain Fv molecules or stabilized diabodies are fused to heavy-chain constant-domains, Fc-regions or parts thereof; (v) Fab fusion molecules, wherein different Fab-fragments are fused together, fused to heavy-chain constant-domains, Fc-regions or parts thereof; and (vi) ScFv- and diabody-based and heavy chain antibodies (e.g., domain antibodies, nanobodies)

wherein different single chain Fv molecules or different diabodies or different heavy-chain antibodies (e.g. domain antibodies, nanobodies) are fused to each other or to another protein or carrier molecule fused to heavy-chain constant-domains, Fc-regions or parts thereof.

Examples of IgG-like molecules with complementary CH3 domain molecules include but are not limited to the Triomab/Quadroma molecules (Trion Pharma/Fresenius Biotech; Roche, WO2011069104), the so-called Knobs-into-Holes molecules (Genentech, WO9850431), CrossMAbs (Roche, WO2011117329) and the electrostatically-matched molecules (Amgen, EP1870459 and WO2009089004; Chugai, US201000155133; Oncomed, WO2010129304), the LUZ-Y molecules (Genentech, Wranik et al. J. Biol. Chem. 2012, 287(52): 43331-9, doi: 10.1074/jbc.M112.397869. Epub 2012 Nov 1), DIG-body and PIG-body molecules (Pharmabcine, WO2010134666, WO2014081202), the Strand Exchange Engineered Domain body (SEEDbody) molecules (EMD Serono, WO2007110205), the Biclonics molecules (Merus, WO2013157953), FcΔAdp molecules (Regeneron, WO201015792), bispecific IgG1 and IgG2 molecules (Pfizer/Rinat, WO11143545), Azymetric scaffold molecules (Zymeworks/Merck, WO2012058768), mAb-Fv molecules (Xencor, WO2011028952), bivalent bispecific antibodies (WO2009080254) and the DuoBody® molecules (Genmab A/S, WO2011131746).

Examples of recombinant IgG-like dual targeting molecules include but are not limited to Dual Targeting (DT)-Ig molecules (WO2009058383), Two-in-one Antibody (Genentech; Bostrom, et al 2009. Science 323, 1610–1614.), Cross-linked Mabs (Karmanos Cancer Center), mAb2 (F-Star, WO2008003116), Zybody molecules (Zyngenia; LaFleur et al. MAbs. 2013 Mar-Apr;5(2):208-18), approaches with common light chain (Crucell/Merus, US7,262,028), κλBodies (NovImmune, WO2012023053) and CovX-body (CovX/Pfizer; Doppalapudi, V.R., et al 2007. Bioorg. Med. Chem. Lett. 17,501–506.).

Examples of IgG fusion molecules include but are not limited to Dual Variable Domain (DVD)-Ig molecules (Abbott, US7,612,181), Dual domain double head antibodies (Unilever; Sanofi Aventis, WO20100226923), IgG-like Bispecific molecules (ImClone/Eli Lilly, Lewis etal. Nat Biotechnol. 2014 Feb;32(2):191-8), Ts2Ab (MedImmune/AZ; Dimasi et al. J Mol Biol. 2009 Oct 30;393(3):672-92) and BsAb molecules (Zymogenetics, WO2010111625), HERCULES molecules (Biogen Idec, US007951918), scFv fusion molecules (Novartis), scFv fusion molecules (Changzhou Adam Biotech Inc, CN 102250246) and TvAb molecules (Roche, WO2012025525, WO2012025530).

Examples of Fc fusion molecules include but are not limited to ScFv/Fc Fusions (Pearce et al., Biochem Mol Biol Int. 1997 Sep;42(6):1179-88), SCORPION molecules (Emergent BioSolutions/Trubion, Blankenship JW, et al. AACR 100 th Annual meeting 2009 (Abstract # 5465); Zymogenetics/BMS, WO2010111625), Dual Affinity Retargeting Technology (Fc-DART) molecules (MacroGenics,

WO2008157379, WO2010080538) and Dual(ScFv)2-Fab molecules (National Research Center for Antibody Medicine – China).

Examples of Fab fusion bispecific antibodies include but are not limited to F(ab)2 molecules (Medarex/AMGEN; Deo et al J Immunol. 1998 Feb 15;160(4):1677-86.), Dual-Action or Bis-Fab molecules (Genentech, Bostrom, et al 2009. Science 323, 1610–1614.), Dock-and-Lock (DNL) molecules (ImmunoMedics, WO2003074569, WO2005004809), Bivalent Bispecific molecules (Biotecnol, Schoonjans, J Immunol. 2000 Dec 15;165(12):7050-7.) and Fab-Fv molecules (UCB-Celltech, WO 2009040562 A1).

Examples of ScFv-, diabody-based and domain antibodies include but are not limited to Bispecific T Cell Engager (BiTE) molecules (Micromet, WO2005061547), Tandem Diabody molecules (TandAb) (Affimed) Le Gall et al., Protein Eng Des Sel. 2004 Apr;17(4):357-66.), Dual Affinity Retargeting Technology (DART) molecules (MacroGenics, WO2008157379, WO2010080538), Single-chain Diabody molecules (Lawrence, FEBS Lett. 1998 Apr 3;425(3):479-84), TCR-like Antibodies (AIT, ReceptorLogics), Human Serum Albumin ScFv Fusion (Merrimack, WO2010059315) and COMBODY molecules (Epigen Biotech, Zhu et al. Immunol Cell Biol. 2010 Aug;88(6):667-75.), dual targeting nanobodies (Ablynx, Hmila et al., FASEB J. 2010) and dual targeting heavy chain only domain antibodies.

The bispecific antibody used in the compositions of the present invention can be of any isotype. Exemplary isotypes include but are not limited to either of the human IgG1, IgG2, IgG3, and IgG4 isotypes. Preferably, bispecific antibodies may be selected to be of the human IgG1 isotype, as shown in the examples. Either of the human light chain constant regions, kappa or lambda, may be used. In one embodiment, both heavy chains of an antibody of the present invention are of the IgG1 isotype. In one embodiment, the two heavy chains of a bispecific antibody are of the IgG1 and IgG4 isotypes, respectively. Preferably, bispecific antibodies may be selected to be of the human IgG1 isotype, as shown in the examples. Optionally, and preferably, the heavy chain and Fc sequences thereof of the selected isotype, may be modified in the hinge and/or CH3 region as described herein to enable the generation of bispecific antibodies and introduce inertness.

In one aspect, the bispecific antibody of the invention comprises an Fc-region comprising a first heavy chain with a first Fc sequence comprising a first CH3 region, and a second heavy chain with a second Fc sequence comprising a second CH3 region, wherein the sequences of the first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions. More details on these interactions and how they can be achieved are provided in WO2011131746 and WO2013060867 (Genmab), which are hereby incorporated by reference.

As described further herein, a stable bispecific CD3xB7H4 antibody can be obtained at high yield on the basis of one B7H4 antibody and one CD3 antibody, each composed of two identical heavy chains and two identical light chains, each antibody containing only a few, fairly conservative, (asymmetrical) mutations in the CH3 regions. Asymmetrical mutations mean that the sequences of said first and second CH3 regions contain one or more amino acid substitutions at non-identical positions.

Antigen-binding region capable of binding CD3

As said, the invention provides a pharmaceutical formulation of or unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3. Furthermore, the invention provides a pharmaceutical formulation of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region capable of binding CD3, is capable of binding human CD3ε (epsilon), such as human CD3ε (epsilon) as specified in SEQ ID NO: 13. Such antigen-binding region is capable of binding human CD3ε (epsilon), as presented on a T cell, such as a primary human T cell.

Said antibody according to the invention may be an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 16 or of SEQ ID NO. 17, and, optionally,

a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22.

CDR1, CDR2 and CDR3 regions can be identified from variable heavy and light chain regions using methods known in the art. The CDR regions from said variable heavy and light chain regions can be annotated according to IMGT (see Lefranc MP. et al., Nucleic Acids Research, 27, 209-212, 1999] and Brochet X. Nucl. Acids Res. 36, W503-508 (2008)). Hence, also disclosed are antibodies comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs.: 18, 19 and 20 or 18, 19 and 21 respectively; and, optionally

a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively.

Further disclosed are antibodies comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding

region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 16, or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 16; and, optionally

5 a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22 or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 22.

Such antigen-binding regions that are capable of binding human CD3 have been described i.a. in
10 WO2015001085, and WO2017009442. Further antigen-binding regions that are capable of binding human CD3 are disclosed and described in WO2015001085 and WO2017009442, which can be further contemplated and serve as the basis for generating antibodies in accordance with the current invention, which are incorporated by reference herein.

15 The said antibody in accordance with the invention, may bind with an equilibrium dissociation constant K_D between the antigen-binding region that binds to human CD3, and human CD3 is within the range of 1 – 1000 nM.

The said antibody in accordance with the invention, may bind with a equilibrium dissociation constant
20 K_D between the antigen-binding region that binds to human CD3, and human CD3 is within the range of 1 – 100 nM, such as within the range of 5 – 100 nM, within the range of 10 – 100 nM, within the range of 1 – 80 nM, within the range of 1 – 60 nM within the range of 1 – 40 nM, within the range of 1 – 20 nM, within the range of 5 – 80 nM, within the range of 5 – 60 nM, within the range of 5 – 40 nM, within the range of 5 – 20 nM, within the range of 10 – 80 nM, within the range of 10 – 60 nM,
25 within the range of 10 – 40 nM, or such as within the range of 10 – 20 nM. An exemplary and suitable antigen-binding region comprises a heavy chain variable region (VH) of SEQ ID NO: 16 and a light chain variable region (VL) regions of SEQ ID NO: 22. Such variable regions have been described i.a. in WO2015001085.

In another aspect of the invention, said antibody has a lower binding affinity for human CD3ε than an
30 antibody having an antigen-binding region comprising a VH sequence as set forth in SEQ ID NO: 16, and a VL sequence as set forth in SEQ ID NO: 22, preferably wherein said affinity is at least 5-fold lower, such as at least 10-fold lower, e.g. at least 20-fold lower, at least 30 fold lower, at least 40 fold lower, at least 45 fold lower or such as at least 50-fold lower.

In another aspect of the invention, said antibody may bind with an equilibrium dissociation constant

K_D between the antigen-binding region that binds to human CD3, and human CD3 antigen-binding which is within the range of 200 – 1000 nM, such as within the range of 300 – 1000 nM, within the range of 400 – 1000 nM, within the range of 500 – 1000 nM, within the range of 300 – 900 nM within the range of 400 – 900 nM, within the range of 400 – 700 nM, within the range of 500 – 900 nM, within
5 the range of 500 – 800 nM, within the range of 500 – 700 nM, within the range of 600 – 1000 nM, within the range of 600 – 900 nM, within the range of 600 – 800 nM, or such as within the range of 600 – 700 nM. An exemplary and suitable antigen-binding region comprises a heavy chain variable region (VH) of SEQ ID NO: 16 or of SEQ ID NO. 17, and, a light chain variable region (VL) regions of SEQ ID NO: 22. Such variable regions have been described i.a. in WO2017009442.

10 Said binding affinity can be determined by biolayer interferometry, optionally as set forth in Example 4 herein. Hence, the antibody according to the invention having a binding affinity to human CD3 as defined herein, may have the binding affinity determined using biolayer interferometry comprising the steps of:

I) immobilizing the antibody at an amount of 1 μg/mL for 600 seconds on an anti-
15 human IgG Fc Capture biosensor;

II) determining association over a time period of 1000 seconds and dissociation over a time period of 2000 seconds of human recombinant soluble CD3ε (CD3E27-GSKa) (mature protein of SEQ ID NO: 13) using a 3-fold dilution series ranging from 1.40 nM to 1000 nM.

20 III) referencing the data to a buffer control (0 nM).

Furthermore, said binding affinity may be determined using an antibody such as a monospecific, bivalent antibody, such as an antibody which is a full length IgG1.

Hence, in a further embodiment, the antibody according to the invention is an antibody, wherein

the antigen-binding region that binds to CD3 comprises a heavy chain variable (VH) region, as
25 defined herein, comprising a CDR1 sequence, a CDR2 sequence and a CDR3 sequence, when compared to a heavy chain variable (VH) region comprising the sequence set forth in SEQ ID NO: 16 has an amino acid substitution being at a position selected from the group consisting of: T31, N57, H101, G105, S110 and Y114, the positions being numbered according to the sequence of SEQ ID NO: 16; and

30 the wild type light chain variable (VL) region comprises the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO: 23, GTN and SEQ ID NO: 24, respectively.

In particular, the antibody according to the invention is an antibody, wherein the antigen-binding

region that binds to CD3 comprises in the heavy chain variable (VH) region as defined herein comprises a substitution selected from the group consisting of: T31M, T31P, N57E, H101G, H101N, G105P, S110A, S110G, Y114M, Y114R, Y114V.

Furthermore, the antibody according to the invention is an antibody wherein the antigen-binding region that binds to CD3 comprises a heavy chain variable region as defined herein having at the amino acid position 31 an M or P, or at the amino acid position 57 an E, or at the amino acid position 101 a G or N, or at the amino acid 105 a P, or at the amino acid position 110 and A or G, or at the amino acid position 114 an M, R or V, said positions corresponding with the amino acid position numbering of the heavy chain variable (VH) region having the sequence set forth in SEQ ID NO: 16.

Still further, the antibody according to the invention is an antibody wherein the CDR1, CDR2 and CDR3 of the heavy chain variable (VH) region of the antigen-binding region that binds to CD3 as defined herein comprises, in total, at the most 1, 2, 3, 4 or 5 amino acid substitutions, when compared with the CDR1, CDR2 and CDR3 of the sequences of SEQ ID NO: 16, said amino acid substitutions comprising preferably amino acid substitutions as defined above.

Further disclosed are pharmaceutical formulations comprising antibodies comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 17, or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 17; and, optionally

a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22 or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 22.

Antigen-binding region capable of binding B7H4

In particular, the invention provides pharmaceutical compositions or unit dosage forms containing an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said human B7H4 is human B7H4 of SEQ ID NO. 1. Preferably, said antibody in accordance with the invention comprises an antigen-binding region capable of binding to human CD3ε (epsilon) as specified in SEQ ID NO: 13, and an antigen-binding region capable of binding human B7H4 of SEQ ID NO. 1.

In particular, the antibody used in the compositions of the present invention is an antibody wherein

said antigen-binding region capable of binding to human B7H4 is capable of binding to the extracellular domain of human B7H4. Preferably, said B7H4 is expressed on a cell, more preferably a human cell.

In a further embodiment, the antibody used in the compositions of the present invention is an antibody wherein said antigen-binding region capable of binding to human B7H4 is capable of binding
5 to the IgC-like constant region of human B7H4. In another further embodiment, the antibody according to the invention is an antibody wherein said antigen-binding region capable of binding to human B7H4 is capable of binding to B7H3-IgV/B7H4-IgC . B7H3-IgV/B7H4-IgC represents a fusion between human B7H3 and B7H4, wherein the B7H3 IgV-like domain is fused with the B7H4 IgC-like domain, corresponding with SEQ ID NO. 11. Said B7H3-IgV/B7H4-IgC being expressed by a cell such
10 as described in the example 7herein. In still another further embodiment, the antibody according to the invention is an antibody wherein said antigen-binding region capable of binding to human B7H4 is not capable of binding to B7H4-IgV/B7H3-IgC. B7H4-IgV/B7H3-IgC represents a fusion between human B7H3 and B7H4, wherein the B7H4 IgV-like domain is fused with the B7H3 IgC-like domain, corresponding with SEQ ID NO. 10. Said B7H4-IgV/B7H3-IgC being expressed by a cell such as
15 described in the example 7 herein.

Suitable antigen-binding regions capable of binding to human B7H4, that are contemplated according to the invention as described herein comprise:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively
20 of SEQ ID NO. 33;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO.
25 36 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 40;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 43 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 47;

30 e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 50 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO.54; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 31 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 65 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 69.

In a preferred embodiment the antibody of the pharmaceutical formulation of the invention comprises an antigen-binding regions capable of binding to human B7H4 comprising a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33.

CDR1, CDR2 and CDR3 regions can be identified from variable heavy and light chain regions using methods known in the art. The CDR regions from said variable heavy and light chain regions can be annotated according to IMGT (see Lefranc MP. et al., Nucleic Acids Research, 27, 209-212, 1999] and Brochet X. Nucl. Acids Res. 36, W503-508 (2008)). Hence, suitable antigen-binding regions capable of binding to human B7H4, that are contemplated according to the invention as described herein comprise:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35 ;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 37, 38 and 39, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 41, DTS and SEQ ID NO. 42;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 44, 45 and 46, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 48, YTS and SEQ ID NO. 49;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 51, 52 and 53, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 55, GAS and SEQ ID NO. 56; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively

of SEQ ID NOs.: 26, 32 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 66, 67 and 68, and a variable light chain region comprising the CDR1, CDR2 and
5 CDR3 respectively of SEQ ID NO. 70, GAS and SEQ ID NO. 71.

In a preferred embodiment the antibody of the pharmaceutical formulation of the invention comprises an antigen-binding regions capable of binding to human B7H4 comprising a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID
10 NO. 34, GAS and SEQ ID NO. 35.

Still further suitable antigen-binding regions capable of binding to human B7H4, that are contemplated according to the invention as described herein comprise:

a) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ ID NO. 33 ;

15 b) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33;

c) a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40;

d) a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ
20 ID NO. 47 ;

e) a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; or

f) a variable heavy chain (VH) region of SEQ ID NO. 31 : and a variable light chain region of SEQ ID NO. 33

25 g) a variable heavy chain (VH) region of SEQ ID NO. 65 : and a variable light chain region of SEQ ID NO. 69.

In a preferred embodiment the antibody of the pharmaceutical formulation of the invention comprises an antigen-binding regions capable of binding to human B7H4 comprising a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33.

30

Optionally, said antigen-binding regions that binds to B7H4 comprise heavy and light chain variable

regions (VH) having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity with:

a) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ ID NO. 33;

5 b) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33 ;

c) a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40;

d) a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ 10 ID NO. 47;

e) a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; or

f) a variable heavy chain (VH) region of SEQ ID NO. 31 : and a variable light chain region of SEQ ID NO. 33

15 g) a variable heavy chain (VH) region of SEQ ID NO. 65 : and a variable light chain region of SEQ ID NO. 69.

In a preferred embodiment said antigen-binding regions that binds to B7H4 comprise heavy and light chain variable regions (VH) having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity with a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain 20 region of SEQ ID NO. 33.

The antibody according to the invention may have an antigen-binding region capable of binding to B7H4 having a binding affinity to human B7H4 that corresponds to a K_D value of 5E-7 M or less, such as 1E-7 M or less, such as with a binding affinity corresponding to a K_D value which is within the range 25 of 5E-7 to 2E-10 M, such as within the range of 2E-7 to 1E-10 M or 1E-7 to 5E-9 M.

Said binding affinity can be determined by biolayer interferometry, optionally as set forth in Example 3 herein. Hence, the antibody according to the invention having a binding affinity to human B7H4 as defined herein, may have the binding affinity determined using biolayer interferometry comprising the steps of:

30 I) immobilizing the antibody at an amount of 1 µg/mL for 600 seconds on an anti-human IgG Fc Capture biosensor;

II) determining association over a time period of 300 seconds and dissociation over a time period of 1000 seconds of human recombinant His tagged B7H4 protein (Sino Biological cat no 10738-H08H; a protein expressed from a construct of DNA sequence encoding the human VTCN1(Uniprot accession no. Q7Z7D3) (Phe29-Ala258) with a C-terminal polyhistidine tag) using a 2-fold dilution series ranging from 1.56 nM to 100 nM.

III) referencing the data to a buffer control (0 nM).

Furthermore, said binding affinity may be determined using an antibody such as a monospecific, bivalent antibody, such as an antibody which is a full length IgG1.

In a further embodiment, an antibody in accordance with the invention is provided, comprising an antigen region capable of binding to human B7H4, wherein said antigen-binding region is capable of crossblocking:

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 29 and a variable light chain region of SEQ ID NO. 33 ; and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40; and

wherein said antigen-binding region is not capable of crossblocking

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47;

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 65 and a variable light chain region of SEQ ID NO. 69.

In still another further embodiment, said antibody in accordance with the invention, comprises an antigen region capable of binding to human B7H4, said antigen-binding region capable of crossblocking

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47 ;

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54, and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 65 and a variable light chain region of SEQ ID NO. 69;

and wherein said antigen-binding region is not capable of crossblocking an antibody comprising

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 29 and a variable light chain region of SEQ ID NO. 33; and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40.

In particular, “cross-blocking”, or the ability of an antibody according to the invention to block binding of another antibody to B7H4, is defined as the ability of a first antibody bound to B7H4 to block binding of a second antibody to the B7H4 bound to the first antibody. Crossblocking can be determined using an assay as described in example 5. Such crossblocking can also be determined e.g. in a procedure comprising the steps of:

i) providing a set of samples, each sample comprising an antibody which binds to B7H4;
ii) immobilizing a first antibody from the set of samples at an amount of 20 µg/mL for 600 seconds on Amine Reactive 2nd Generation biosensor (AR2G);
iii) loading the ARG2 biosensor with immobilized antibody with human B7H4 (100 nM of human recombinant His tagged B7H4 protein (Sino Biological cat no 10738-H08H; a protein expressed from a construct of DNA sequence encoding the human VTCN1(Uniprot accession no. Q7Z7D3) (Phe29-Ala258) with a C-terminal polyhistidine tag)
iv) determining the association of a second antibody from the set of samples at an amount of 10 µg/mL for 300 seconds.

When the second antibody is not capable of association, the first antibody is considered to cross-block the second antibody. The skilled person will be familiar with suitable technologies for determining the ability of an antibody to crossblock the binding of another antibody to its target, the present application discloses procedures suitable for determining blocking of binding and displacement. In a further embodiment, crossblocking as described herein is determined as described in Example 5.

In a further embodiment, the antibody in accordance with the invention, having an antigen-binding region capable of binding to human B7H4 complying with a crossblocking feature as described above, wherein said an antigen-binding region capable of binding to human B7H4 is capable of binding to B7H3-IgV/B7H4-IgC (SEQ ID NO. 11), and optionally is not capable of binding to B7H4-IgV/B7H3-IgC (SEQ ID NO. 10).

CD3 and B7H4 antigen-binding region combinations

The present disclosure further provides pharmaceutical compositions or unit dosage forms comprising an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to
5 CD3 comprises

a heavy chain variable region (VH) comprising,the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 16, and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22. and

wherein the antigen-binding region capable of binding to B7H4 comprises:

10 a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33 ;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively
15 of SEQ ID NO. 33 ;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 36 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 40 ;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO.
20 43 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 47 ;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 50 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO.54 ; or

25 f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 31 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 65 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively
30 of SEQ ID NO. 69.

Again, CDR1, CDR2 and CDR3 regions can be identified from variable heavy and light chain regions using methods known in the art. The CDR regions from said variable heavy and light chain regions can

be annotated according to IMGT (see Lefranc MP. et al., Nucleic Acids Research, 27, 209-212, 1999] and Brochet X. Nucl. Acids Res. 36, W503-508 (2008)). In preferred embodiments the CDR regions from the variable heavy and light chain regions of the invention are annotated according to IMGT.

The present disclosure further provides pharmaceutical compositions and unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising,the CDR1, CDR2, and CDR3 regions of SEQ ID NO. 17, and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22. and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33 ;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33 ;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 36 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 40 ;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 43 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 47 ;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 50 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO.54 ; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 31 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 65 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 69.

In preferred embodiments the present invention provides pharmaceutical compositions and unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises:

5 a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO. 17, and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO. 22, and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID

10 NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33.

Also, the present disclosure further provides pharmaceutical compositions and unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region capable of

15 binding to CD3 comprises:

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs.: 18, 19 and 20 respectively; and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively; and

wherein the antigen-binding region capable of binding to B7H4 comprises:

20 a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35 ;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and

25 CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35 ;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 37, 38 and 39, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 41, DTS and SEQ ID NO. 42 ;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively

30 of SEQ ID NOs.: 44, 45 and 46, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 48, YTS and SEQ ID NO. 49 ;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 51, 52 and 53, and a variable light chain region comprising the CDR1, CDR2 and

CDR3 respectively of SEQ ID NO. 55, GAS and SEQ ID NO. 56 ; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 32 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35

5 g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 66, 67 and 68, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 70, GAS and SEQ ID NO. 71.

The present disclosure further provides pharmaceutical compositions and unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-
10 binding region capable of binding to human CD3, wherein
the antigen-binding region capable of binding to CD3 comprises:

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs.: 18, 19 and 21 respectively; and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively; and

15 wherein the antigen-binding region capable of binding to B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35 ;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively
20 of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35 ;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 37, 38 and 39, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 41, DTS and SEQ ID NO. 42 ;

25 d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 44, 45 and 46, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 48, YTS and SEQ ID NO. 49 ;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 51, 52 and 53, and a variable light chain region comprising the CDR1, CDR2 and
30 CDR3 respectively of SEQ ID NO. 55, GAS and SEQ ID NO. 56 ; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 32 and 28, and a variable light chain region comprising the CDR1, CDR2 and

CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 66, 67 and 68, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 70, GAS and SEQ ID NO. 71.

5 In preferred embodiments the present invention provides pharmaceutical compositions and unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of SEQ 10 ID NOs.: 18, 19 and 21 respectively; and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively; and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 15 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35.

Further disclosed are pharmaceutical compositions and unit dosage forms of antibodies comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises :

20 a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 16 and, a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22; and

wherein the antigen-binding region capable of binding to B7H4 comprises an antigen-binding regions that bind to B7H4 comprise heavy and light chain variable regions (VH) having:

a) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ 25 ID NO. 33 ;

b) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33 ;

c) a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40 ;

30 d) a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47 ;

e) a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ

ID NO.54 ; or

f) a variable heavy chain (VH) region of SEQ ID NO. 31 : and a variable light chain region of SEQ ID NO. 33

g) a variable heavy chain (VH) region of SEQ ID NO. 65 : and a variable light chain region of SEQ
5 ID NO. 69.

Still further disclosed are pharmaceutical compositions and unit dosage forms of antibodies comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises:

10 a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 17 and, a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22; and

wherein the antigen-binding region capable of binding to B7H4 comprises antigen-binding heavy and light chain variable regions (VH) having:

a) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ
15 ID NO. 33 ;

b) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33 ;

c) a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40 ;

20 d) a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47 ;

e) a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54 ; or

f) a variable heavy chain (VH) region of SEQ ID NO. 31 : and a variable light chain region of SEQ ID
25 NO. 33

g) a variable heavy chain (VH) region of SEQ ID NO. 65 : and a variable light chain region of SEQ ID NO. 69.

In preferred embodiments the present invention provides pharmaceutical compositions and unit dosage forms of an antibody comprising an antigen-binding region capable of binding to human B7H4
30 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 17 and, a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22; and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33.

In a further embodiment, in such a bispecific antibody, said antigen binding region capable of binding to human B7H4 is comprised in an heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a kappa light chain constant region; and said antigen binding region capable of binding to human CD3 is comprised in a heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a lambda light chain constant region. More preferably, in such a CD3xB7H4 bispecific antibody, one IgG1 heavy chain constant region is as defined in SEQ ID NO. 60 and the other is as defined in SEQ ID NO. 61, and wherein said kappa light chain constant region is as defined in SEQ ID NO. 63 and said lambda light chain constant region is as defined in SEQ ID NO. 64. It is understood that optionally, of said IgG1 heavy chain constant regions as defined in SEQ ID NO. 60 and 61, the terminal lysines can be deleted.

Accordingly, in a preferred embodiment of the invention the CD3xB7H4 bispecific antibody of the pharmaceuticl formulation of the unit dosage form comprises a CD3 binding region comprising:

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 17 and, a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22, and further an IgG1 heavy chain constant region as defined in SEQ ID NO. 60 and a lambda light chain constant region is as defined in SEQ ID NO. 64,

and a B7H4 binding region comprising:

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 29 and, a light chain variable region (VL) comprising the sequence of SEQ ID NO: 33, and further an IgG1 heavy chain constant region as defined in SEQ ID NO. 61 and a kappa light chain constant region is as defined in SEQ ID NO. 63, optionally, wherein said IgG1 heavy chain constant regions as defined in SEQ ID NO. 60 and 61 may have the terminal lysines deleted.

As will be well-known to the skilled person, each antigen-binding region of an antibody generally comprise a heavy chain variable region (VH) and a light chain variable region (VL), and each of the

variable regions comprises three CDR sequences, CDR1, CDR2 and CDR3, respectively, and may comprise four framework sequences, FR1, FR2, FR3 and FR4, respectively. Each antigen-binding region of an antibody may generally comprise a heavy chain variable region (VH) and a light chain variable region (VL), and each of the variable regions comprises three CDR sequences, CDR1, CDR2 and CDR3,
5 respectively, and may comprise four human framework sequences, FR1, FR2, FR3 and FR4, respectively. This structure is preferably also found in the antibodies according to the present invention. Furthermore, the antibodies according to the invention may comprise two heavy chain constant regions (CH), and two light chain constant regions (CL). Examples of constant regions are provided i.a. in SEQ ID NOs. 57-64.

10 In particular embodiments, the antibody used in the pharmaceutical compositions and unit dosage forms of invention comprises a first and a second heavy chain, such as a first and second heavy chain each comprising at least a hinge region, a CH2 and CH3 region. Stable, heterodimeric antibodies can be obtained at high yield for instance by so-called Fab-arm exchange as provided in WO 2008/119353 and WO 2011/131746, on the basis of two homodimeric starting proteins containing only a few,
15 asymmetrical mutations in the CH3 regions. Hence, in some embodiments of the invention, the antibody comprises a first heavy chain wherein at least one of the amino acids at the positions corresponding to positions selected from the group consisting of T366, L368, K370, D399, F405, Y407 and K409 in a human IgG1 heavy chain has been substituted, and a second heavy chain wherein at least one of the amino acids in the positions corresponding to a position selected from the group
20 consisting of T366, L368, K370, D399, F405, Y407, and K409 in a human IgG1 heavy chain has been substituted, wherein said substitutions of said first and said second heavy chains are not in the same positions, and wherein the amino acid positions are numbered according to Eu numbering. For example, constant domains having such a substitution are provided i.a. in SEQ ID NO. 58 and 62, which can be compared with SEQ ID NO. 57, which does not have such a substitution.

25 The term “amino acid corresponding to positions” as used herein refers to an amino acid position number in a human IgG1 heavy chain. Corresponding amino acid positions in other immunoglobulins may be found by alignment with human IgG1. Unless otherwise stated or contradicted by context, the amino acids of the constant region sequences are herein numbered according to the EU-index of numbering (described in Kabat, E.A. et al., 1991, Sequences of proteins of immunological interest. 5th
30 Edition - US Department of Health and Human Services, NIH publication No. 91-3242, pp 662, 680, 689). Thus, an amino acid or segment in one sequence that “corresponds to” an amino acid or segment in another sequence is one that aligns with the other amino acid or segment using a standard sequence alignment program such as ALIGN, ClustalW or similar, typically at default settings and has at least 50%, at least 80%, at least 90%, or at least 95% identity to a human IgG1 heavy chain. It is

considered well-known in the art how to align a sequence or segment in a sequence and thereby determine the corresponding position in a sequence to an amino acid position according to the present invention.

In particular embodiments, the invention provides pharmaceutical compositions and unit dosage forms of an antibody, wherein the amino acid in the position corresponding to K409 in a human IgG1 heavy chain is R in said first heavy chain, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain is L in said second heavy chain, or *vice versa*.

In some embodiments, the antibody according to the present invention comprises, in addition to the antigen-binding regions, comprises an Fc region with Fc sequences of the two heavy chains. The first and second Fc sequence may each be of any isotype, including any human isotype, such as an IgG1, IgG2, IgG3, IgG4, IgE, IgD, IgM, or IgA isotype or a mixed isotype. Preferably, the Fc region is a human IgG1, IgG2, IgG3, IgG4 isotype or a mixed isotype, such as a human IgG1 isotype. In some embodiments, it is preferred that the antibody according to the invention is a full-length antibody, most preferably it is of the IgG1 type.

Antibodies according to the present invention may comprise modifications in the Fc region to render the antibody an inert, or non-activating, antibody. Hence, in the antibodies disclosed herein, one or both heavy chains may be modified so that the antibody induces Fc-mediated effector function to a lesser extent relative to an antibody which is identical, except for comprising non-modified first and second heavy chains. The Fc-mediated effector function may be measured by determining Fc-mediated CD69 expression on T cells (i.e. CD69 expression as a result of CD3 antibody-mediated, Fcγ receptor-dependent CD3 crosslinking), by binding to Fcγ receptors, by binding to C1q, or by induction of Fc-mediated cross-linking of FcγRs. In particular, the heavy chain constant sequences may be modified so that the Fc-mediated CD69 expression is reduced by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 99% or 100% when compared to a wild-type (unmodified) antibody, wherein said Fc-mediated CD69 expression is determined in a PBMC-based functional assay, e.g. as described in Example 3 of WO2015001085. Modifications of the heavy and light chain constant sequences may also result in reduced binding of C1q to said antibody. As compared to an unmodified antibody the reduction may be by at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, or 100% and the C1q binding may be determined by ELISA. Further, the Fc region which may be modified so that said antibody mediates reduced Fc-mediated T-cell proliferation compared to an unmodified antibody by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 99% or 100%, wherein said T-cell proliferation is measured in a PBMC-based functional assay.

A wide range of different non-activating antibody formats have been developed in which amino acid

substitutions, and combinations thereof, have been introduced in the constant heavy chain region of an IgG1 isotype antibody to eliminate Fc-mediated effector functions (e.g. Chiu et al., Antibodies 2019 Dec; 8(4): 55; Liu et al., Antibodies, 2020 Nov 17;9(4):64; 29(10):457-66; Shields et al., J Biol Chem,. 2001 Mar 2;276(9):6591-604).

5 Examples of amino acid positions that may be modified, e.g. in an IgG1 isotype antibody, include positions L234 and L235. Hence, the antibody according to the invention may comprises a first and a second heavy chain, and wherein in both the first and the second heavy chain, the amino acid residues at the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to Eu numbering are F and E, respectively. It is understood that in addition to modifications of amino 10 acid positions L234 and L235, further positions may be modified.

In addition, a D265A amino acid substitution can decrease binding to all Fcγ receptors and prevent ADCC (Shields et al., 2001, J. Biol. Chem. (276):6591-604). Therefore, the antibody according to the invention may comprise a first and a second heavy chain, wherein in both the first and the second heavy chain, the amino acid residue at the position corresponding to position D265 in a human IgG1 15 heavy chain according to Eu numbering is A. Further embodiments of the invention provide antibodies wherein, in at least one, such as in both, of said first and second heavy chains the amino acids in the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain, are F, E, and A, respectively. In the present application antibodies, which have the combination of three amino acid substitutions L234F, L235E and D265A and in addition the K409R or the F405L mutation disclosed 20 herein above may be termed with the suffix “FEAR” or “FEAL”, respectively.

An amino acid sequence of a wild type IgG1 heavy chain constant region is identified herein as SEQ ID NO: 57. Consistent with the embodiments disclosed above, the antibody of the invention may comprise an IgG1 heavy chain constant region carrying the F405L substitution and may have the amino acid sequence set forth in SEQ ID NO: 58 and/or an IgG1 heavy chain constant region carrying the 25 K409R substitution and may have the amino acid sequence set forth in SEQ ID NO: 62 .

An amino acid sequence of an IgG1 heavy chain constant region carrying the L234F, L235E and D265A substitutions is identified herein as SEQ ID NO: 59. An amino acid sequence of an IgG1 heavy chain constant region carrying the L234F, L235E, D265A and F405L substitutions is identified herein as SEQ ID NO: 60. An amino acid sequence of an IgG1 heavy chain constant region carrying the L234F, L235E, 30 D265A and K409R substitutions is identified herein as SEQ ID NO: 61.

The constant region sequences listed in SEQ ID NOs. 57-62 list a terminal lysine (K), such sequences were used in the example section herein. The origin of this lysine is a naturally occurring sequence found in humans from which these Fc regions are derived. During cell culture production of

recombinant antibodies, this terminal lysine can be cleaved off by proteolysis by endogenous carboxypeptidase(s), resulting in a constant region having the same sequence but lacking the C-terminal lysine. For manufacturing purposes of antibodies, the DNA encoding this terminal lysine can be omitted from the sequence such that antibodies are produced without the lysine. Antibodies
5 produced from nucleic acid sequences that either do, or do not encode a terminal lysine are substantially identical in sequence and in function since the degree of processing of the terminal lysine is typically high when e.g. using antibodies produced in CHO-based production systems (Dick, L.W. et al. Biotechnol. Bioeng. 2008;100: 1132–1143). Hence, it is understood that antibodies in accordance with the invention can be generated without encoding or having a terminal lysine such as listed herein.
10 For manufacturing purposes, antibodies can thus be generated without having a terminal lysine.

The present invention further provides pharmaceutical compositions and unit dosage forms of an antibody, wherein

a) the antigen-binding region capable of binding to B7H4 is human, and
b) the antigen-binding region capable of binding to CD3, is humanized.

15 Also, the invention provides pharmaceutical compositions and unit dosage forms of an antibody, wherein

a) the antigen-binding region capable of binding to B7H4 is human, and/or
the antigen-binding region capable of binding to CD3, is humanized

In some embodiments of the invention, the antibody comprises a kappa (κ) light chain. The sequence
20 of particular embodiments of the invention concerning bispecific antibodies, the kappa light chain comprises the CDR1, -2 and -3 sequences of a B7H4 antibody light chain as disclosed above.

In further embodiments of the invention, the antibody comprises a lambda (λ) light chain. In particular embodiments of the invention concerning bispecific antibodies, the lambda light chain comprises the CDR1, -2 and -3 sequences of a CD3 antibody light chain as disclosed above, in particular a the CDR1,
25 -2 and -3 sequences of a CD3 antibody having reduced affinity for CD3 as disclosed above. The amino acid sequence of a kappa light chain constant region is included herein as SEQ ID NO: 63 and the amino acid sequence of a lambda light chain constant region is included herein as SEQ ID NO: 64.

In particular embodiments, the antibody comprises a lambda (λ) light chain and a kappa (κ) light chain; e.g. an antibody with a heavy chain and a lambda light chain which comprise the binding region
30 capable of binding to CD3, and a heavy chain and a kappa light chain which comprise the binding region capable of binding to B7H4.

Hence, in a further embodiment, in a bispecific antibody as defined herein, said antigen binding region capable of binding to human B7H4 is comprised in a heavy chain and a light chain, said heavy chain

comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a kappa light chain constant region; and said antigen binding region capable of binding to human CD3 is comprised in a heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region
5 and a lambda light chain constant region. More preferably, in said bispecific antibody, one IgG1 heavy chain constant region is as defined in SEQ ID NO. 60 and the other is as defined in SEQ ID NO. 61, and said kappa light chain constant region is as defined in SEQ ID NO. 63 and said lambda light chain constant region is as defined in SEQ ID NO. 64. It is understood that said IgG1 heavy chain constant regions as defined in SEQ ID NO. 60 and 61 may have their terminal lysines deleted.

10 ***Binding, cytoxicity and T-cell activation***

Antibodies, such as bispecific antibodies, as described herein that can bind to human CD3 and human B7H4 can advantageously target T cells to human B7H4 expressing cancer cells, thereby inducing T-cell mediated killing of said cancer cells. By having reduced or inert Fc-functionality in such antibodies, as shown in the example section, safe, effective and sufficient antibody can be administered to human
15 patients, while being efficacious against a wide range of cancers varying in B7H4 expression levels.

As said, preferably, the antibody in accordance with the invention is devoid of, or has reduced Fc-mediated effector function, and furthermore, the antibody:

a) is capable of binding to B7H4-expressing human tumor cells as described in Example 9 and 10 herein,
20 b) is capable of mediating concentration-dependent cytotoxicity of B7H4-expressing human tumor cells when using e.g. purified PBMCs or T cells as effector cells when assayed as described in Example 11 and 12 herein,
c) is capable of mediating concentration-dependent cytotoxicity of one or more human B7H4-expressing tumor cell lines selected from the group consisting of MCF-7, MDA-MB-
25 468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650, when using e.g. purified PBMCs or T cells as effector cells when assayed as described in Example 11 and 12 herein,
d) is capable of activating T cells *in vitro* in the presence of B7H4-expressing human tumor cells; e.g. when assayed as described in Example 13 herein,
e) is capable of activating T-cells *in vitro* in the presence of one or more B7H4-expressing
30 human tumor cell lines selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650; e.g. when assayed as described in Example 13 herein,
f) is capable of inducing cytotoxicity of B7H4-expressing human tumor cells; e.g. when

assayed as described in Example 11 and 12 herein, and/or

g) is capable of inducing T cell mediated cytotoxicity in one or more B7H4-expressing human tumor cell lines selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650; e.g. when assayed as described in Example 11 and 12 herein.

Furthermore, the antibody in accordance with the invention may be devoid of, or has reduced Fc-mediated effector function, and, furthermore capable of inducing T-cell mediated cytotoxicity antibody, wherein cytoxicity is assessed in an *in vitro* IC50 assay comprising:

i) providing isolated peripheral blood mononuclear cells (PBMCs), or purified T-cells, from healthy human donor buffy coats,
ii) providing B7H4-expressing tumor cells, such as a human B7H4-expressing tumor cell line selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650;
iii) combining said PBMCs or said purified T-cells with a plurality of samples of said B7H4-expressing tumor cells, wherein the ratio of the number of T-cells from said PBMCs, or said purified T-cells, to the selected tumor cell is 8:1;
iv) providing said antibody in a dilution series to said samples, ranging e.g. from 0.0128 ng/mL to 10,000 ng/mL for a selected human B7H4 expressing tumor cell, and
v) incubating the samples obtained in step iv), e.g. for 72 hours at 37°C; and subsequently,
vi) assessing the viability of the B7H4-expressing tumor cells,
vii) determining the percentage of viable cells for each dilution sample, and
viii) determining the IC50.

Instead of isolated peripheral blood mononuclear cells (PBMCs), purified T-cells may also be provided in step i).

Accordingly, the antibody may have an IC50 in the range of 0.001-2 microgram/ml, wherein the IC50 is determined in an *in vitro* cytotoxicity assay comprising the steps of:

i) providing isolated peripheral blood mononuclear cells (PBMCs) from healthy human donor buffy coats,
ii) providing B7H4-expressing tumor cells, such as a human B7H4-expressing tumor cell line selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, and HCC1954;
iii) combining said PBMCs with a plurality of samples of said B7H4-expressing tumor cells, wherein the ratio of the number of T-cells from said PBMCs to the selected tumor cell is

8:1;

iv) providing said antibody in a dilution series to said samples, ranging e.g. from 0.0128 ng/mL to 10,000 ng/mL for a selected human B7H4 expressing tumor cell, and

v) incubating the samples obtained in step iv), e.g. for 72 hours at 37°C; and subsequently,

5 vi) assessing the viability of the B7H4-expressing tumor cells,

vii) determining the percentage of viable cells for each dilution sample, and

viii) determining the IC50.

Accordingly, the antibody may have an IC50 in the range of 0.001-5 microgram/ml, wherein the IC50 is determined in an *in vitro* cytotoxicity assay comprising the steps of:

10 i) providing isolated peripheral blood mononuclear cells (PBMCs), or purified T-cells, from healthy human donor buffy coats,

ii) providing B7H4-expressing tumor cells, such as a human B7H4-expressing tumor cell line selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650;

15 iii) combining said PBMCs or said purified T-cells with a plurality of samples of said B7H4-expressing tumor cells, wherein the ratio of the number of T-cells from said PBMCs, or said purified T-cells, to the selected tumor cell is 8:1;

iv) providing said antibody in a dilution series to said samples, ranging e.g. from 0.0128 ng/mL to 10,000 ng/mL for a selected human B7H4 expressing tumor cell, and

20 v) incubating the samples obtained in step iv), e.g. for 72 hours at 37°C; and subsequently,

vi) assessing the viability of the B7H4-expressing tumor cells,

vii) determining the percentage of viable cells for each dilution sample, and

viii) determining the IC50.

In one embodiment, the antibody in accordance with the invention may have an IC50 in the range of 25 0.001-5 microgram/ml. In one embodiment, the antibody in accordance with the invention may have an IC50 in the range of 0.001-2 microgram/ml. In another embodiment, the antibody in accordance with the invention may have an IC50 is in the range of 0.001 -0.03 microgram/ml. In still a further embodiment, the IC50 may be in the range of 0.05 – 2 microgram/ml. In yet another further embodiment, the IC50 may be in the range of 0.05 – 5 microgram/ml. Said IC50 may be determined 30 using a method such as described in Example 12.

In a further embodiment, the ability of the antibody in accordance with the invention to mediate T cell activation is determined in an *in vitro* assay comprising the steps of:

i) providing isolated peripheral blood mononuclear cells (PBMCs) from healthy human donor buffy coats,
ii) providing B7H4-expressing tumor cells;
iii) combining PBMCs and B7H4-expressing tumor cells in a plurality of samples, wherein the
5 ratio of the number of PBMCs to tumor cells is 8:1;
iv) providing said antibody in a dilution series to said samples, ranging e.g. from 0.0128 ng/mL to 10,000 ng/mL and
v) incubating the samples, e.g. for 72 hours at 37°C; and
vi) subsequently detecting cytokines.

10 Exemplary cytokines that can be e.g. detected are e.g. IFN-γ, such as e.g. described in example 13. Preferably B7H4-expressing tumor cells are human B7H4-expressing tumors, such as primary tumors, or tumor cell lines selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, and HCC1954.

B7H4 antibodies

15 In another aspect, the present invention relates to a pharmaceutical composition or unit dosage form, comprising

an antibody comprising an antigen-binding region capable of binding to human B7H4, wherein said antigen-binding region capable of binding to human B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO.
20 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

25 c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 31 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and
30 CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and

CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 32 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

5 g) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ ID NO. 33; or

h) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33;

i) a variable heavy chain (VH) region of SEQ ID NO. 31 : and a variable light chain region of SEQ ID
10 NO. 33;

j) having a heavy (VH) and light (VH) chain variable regions having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity with the respective variable heavy chain (VH) region of SEQ ID NO. 25 and the variable light chain region of SEQ ID NO. 33,

wherein the pharmaceutical composition or unit dosage form further comprises a buffering agent,
15 and wherein the pH of the composition is from 4.0 to 8.0.

Such antibodies do not necessarily comprise an antigen-binding region that binds to CD3. Such antibodies may be useful, e.g. in kits and assays for detecting B7H4. Such antibodies may also be useful in the treatment of cancer. Hence, such an antibody may be monospecific antibody binding to B7H4. Such an antibody may be a bivalent antibody.

20 It is preferred that the pH of the pharmaceutical composition or the unit dosage form is from 4.5 to 6.5. In a preferred embodiment, the pH of the pharmaceutical composition or the unit dosage form is from 5.0 to 6.5. In a particularly preferred embodiment, the the pH of the pharmaceutical composition or the unit dosage form is from 5.0 to 6.0. In a preferred embodiment, the buffering agent is selected from the group consisting of histidine, glutamate, and mixtures thereof. In a preferred embodiment,
25 the pharmaceutical composition further comprises c) a non-ionic excipient. In a preferred embodiment, the non-ionic excipient is a sugar or a sugar alcohol. In a preferred embodiment, the non-ionic excipient is selected from sorbitol, sucrose or mixtures thereof. In a preferred embodiment, the non-ionic excipient is present at a concentration of 100 to 300 mM, such as 125-250 mM preferably 250 mM. In a preferred embodiment, the pharmaceutical composition further comprises
30 d) a surfactant. In a preferred embodiment, the surfactant is a polysorbate, preferably polysorbate 20 or 80, most preferably polysorbate 80. In a preferred embodiment, the surfactant is present at a concentration from about 0.005% to 0.4% w/v, such as from about 0.01 to 0.1 % w/v, such as from

about 0.01 to 0.09 % w/v such as from about 0.01 to 0.06 % w/v such as from about 0.01 to 0.05% w/v such as 0.02% w/v or 0.03% w/v or 0.04% w/v or 0.05% w/v, preferably 0.02% w/v. Any feature or embodiments described above for the first aspect of the present invention may likewise apply to this aspect of the present invention.

5 Preferably, such an antibody is an antibody comprising a heavy chain constant region which is a human IgG1 constant region. For example, a heavy chain constant region such as listed in SEQ ID NO. 57-62. A preferred light chain constant region is a kappa light chain, such as listed in SEQ ID NO. 63.

In one embodiment, the antibody of the pharmaceutical composition or unit dosage form provided herein may bind to an epitope or antibody binding region on human B7H4 comprising one or more of

10 the amino acid residues S151, V157, D158, Y159, E164, L166, W173, P175, P177, V179, W181, F199, M208, V210, T222, Y223, V240, E242 and I245; the numbering of each amino acid residue referring to its position in SEQ ID NO: 1. In a further embodiment, the antibody provided herein may bind to an epitope or antibody binding region on human B7H4 comprising one or more of the amino acid residues V157, D158, Y159, E164, L166; the numbering of each amino acid residue referring to its position in

15 SEQ ID NO: 1.

In another embodiment, the antibody provided herein may bind to an epitope or antibody binding region on human B7H4 comprising the amino acid residues S151, V157, D158, Y159, E164, L166, W173, P175, P177, V179, W181, F199, M208, V210, T222, Y223, V240, E242 and I245; the numbering of each amino acid residue referring to its position in SEQ ID NO: 1. In a further embodiment, the antibody

20 provided herein may bind to an epitope or antibody binding region on human B7H4 comprising the amino acid residues V157, D158, Y159, E164, L166; the numbering of each amino acid residue referring to its position in SEQ ID NO: 1.

Based on the results provided in Example 7 herein it is hypothesized, without any wish to be bound by theory, that any one or more of these amino acid residues (i.e. S151, V157, D158, Y159, E164, L166,

25 W173, P175, P177, V179, W181, F199, M208, V210, T222, Y223, V240, E242 and I245) is/are directly involved in binding of the antibody, such as by way of non-covalent interactions; e.g with amino acid residues within the CDR sequences of the antibody.

The amino acid residues comprised by said epitope or antibody binding region and optionally the one or more additional amino acid residues which are indirectly involved in binding may be identified by

30 alanine scanning of human B7H4 having the amino acid sequence set forth in SEQ ID NO: 1 or the extracellular domain sequence of SEQ ID NO: 1. The alanine scanning may in particular be performed as set forth or essentially as set forth in Example 7 herein.

Further, the alanine scanning may be performed by a procedure comprising the steps of:

i) Expressing mutant human B7H4 polypeptides in which amino acid residues in the extracellular domain of human B7H4, except cysteines and alanines, are individually substituted with alanine, and corresponding wild type B7H4 polypeptides individually in human embryonic kidney cells, e.g. HEK 293 cells, such that for each mutant or wild type
5 B7H4 a sample comprising 40-60.000 cells, such as 50.000 cells is provided,

ii) Incubating the cells in each sample with 20 µL of said antibody, wherein said antibody consists of a single heavy chain and a single light chain, which antibody is labelled, e.g. with a suitable label for flow cytometry analysis such as an mNeogreen label, and incubated for an hour at room temperature, and subsequently washing with FACS buffer
10 (e.g. phosphate-buffered saline [PBS; Lonza, cat. no. BE17-517] + 0.1% [w/v] BSA [Roche, cat. no. 10735086001] + 0.02% [w/v] sodium azide [NaN_3; EMELCA Bioscience, cat. no. 41920044-3]) and resuspending the cells in each sample in 30 µL FACS buffer,

iii) Determining, for each sample, the average amount of antibody bound per cell as the geometric mean of the fluorescence intensity (gMFI) for the viable, single cell population
15 in said sample and normalizing the data for each test antibody against the binding intensity of a non-cross blocking B7H4-specific reference antibody using the equation:

$$\boldsymbol{Normalized\ gMFI_{aa\,position} = Log_{10}\left(\frac{gMFI_{Test\,Ab}}{gMFI_{Control\,Ab}}\right)}$$

wherein ‘aa position’ refers to the position that was mutated into an alanine,

wherein the fold-change or Z-score is calculated to express loss or gain of binding of the
20 antibody, according to the calculation:

$$\boldsymbol{Fold\ Change = Log_{10}\left(\frac{Normalized\ gMFI_{ala\ mutant}}{Normalized\ gMFI_{wt}}\right)}$$

wherein amino acid positions for which, upon replacing the amino acid with alanine, there is no loss or gain of binding by a particular antibody will give as result ‘0’, and gain of binding will result in ‘>0’ and loss of binding will result in ‘<0’, and wherein, only B7H4
25 amino acid residues where the Fold Change in binding was lower than the mean Fold Change – 1.5 x SD, where SD is the standard deviation of calculated fold changes from four independent experiments for a particular test antibody, were considered ‘loss of binding mutants’, and, wherein, in case the gMFI of the reference antibody for a particular B7H4 mutant was lower than the mean gMFI - 2.5 x SD of the mean gMFI
30 *Control Ab*, data were excluded from analysis.

Furthermore, such an antibody may also be a bispecific antibody comprising in addition to an antigen-

binding region capable of binding to B7H4 another antigen-binding region. Such another antigen-binding region may be an antigen-binding region capable of binding to human CD3. Such antigen-binding region capable of binding to human CD3 may be antigen-binding regions capable of binding to CD3 as described and disclosed herein.

5 In a further embodiment the pharmaceutical composition or unit dosage form of the invention comprises a bispecific antibody wherein said antigen binding region capable of binding to human B7H4 is comprised in an heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a kappa light chain constant region; and wherein said antigen binding region capable of binding to human CD3 is 10 comprised in a heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a lambda light chain constant region. More preferably, in such a bispecific antibody, one IgG1 heavy chain constant region is as defined in SEQ ID NO. 60 and the other is as defined in SEQ ID NO. 61, and wherein said kappa light chain constant region is as defined in SEQ ID NO. 63 and said lambda light chain constant region 15 is as defined in SEQ ID NO. 64. It is understood that optionally, of said IgG1 heavy chain constant regions as defined in SEQ ID NO. 60 and 61, the terminal lysines can be deleted.

A highly preferred bispecific antibody of the pharmaceutical composition or unit dosage form of the invention is as described and used in the example section, and is referred to as BsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR.

20 Hence, In a preferred embodiment, the pharmaceutical composition or unit dosage form comprises a bispecific antibody capable of binding human CD3 and human B7H4 is provided comprising:

- a first heavy chain and a first light chain which comprise the binding region capable of binding to human CD3, wherein said first heavy chain comprises a heavy chain variable region as defined by SEQ ID NO: 17 and a human IgG1 heavy chain constant region as defined herein, 25 and wherein said first light chain comprises a light chain variable region as defined by SEQ ID NO: 22 and a human lamba light chain constant region; and
- a second heavy chain and a second light chain which comprise the binding region capable of binding to human B7H4, wherein said second heavy chain comprises a heavy chain variable region as defined by SEQ ID NO: 29 and a human IgG1 heavy chain constant region as defined 30 herein, and wherein said second light chain comprises a light chain variable region as defined by SEQ ID NO: 33 and a human kappa light chain constant region.

It is understood that the human IgG1 heavy chain constant regions as defined herein may encompass substitutions as defined herein (e.g. FEAR/FEAL), or the like. It is also understood that the human IgG1

heavy chain constant region may have its terminal lysine (K) deleted.

In a further preferred embodiment, the pharmaceutical composition or unit dosage form comprises a bispecific antibody capable of binding human CD3 and human B7H4 is provided comprising:

- a first heavy chain and a first light chain which comprise the binding region capable of binding to human CD3, wherein said first heavy chain comprises a heavy chain variable region as defined by SEQ ID NO: 17 and a heavy chain constant region as defined by SEQ ID NO: 60, and wherein said first light chain comprises a light chain variable region as defined by SEQ ID NO: 22 and a light chain constant region as defined by SEQ ID NO: 64; and
- a second heavy chain and a second light chain which comprise the binding region capable of binding to human B7H4, wherein said second heavy chain comprises a heavy chain variable region as defined by SEQ ID NO: 29 and a heavy chain constant region as defined by SEQ ID NO: 61, and wherein said second light chain comprises a light chain variable region as defined by SEQ ID NO: 33 and a light chain constant region as defined by SEQ ID NO: 63.

Likewise, it is understood that the human IgG1 heavy chain constant region may have its terminal lysine (K) deleted.

In yet another further preferred embodiment, the pharmaceutical composition or unit dosage form comprises a bispecific antibody capable of binding human CD3 and human B7H4 is provided comprising:

- a first heavy chain and a first light chain which comprise the binding region capable of binding to human CD3, wherein said first heavy chain consists of a heavy chain variable region as defined by SEQ ID NO: 17 and a heavy chain constant region as defined by SEQ ID NO: 60, and wherein said first light chain consists of a light chain variable region as defined by SEQ ID NO: 22 and a light chain constant region as defined by SEQ ID NO: 64; and
- a second heavy chain and a second light chain which comprise the binding region capable of binding to human B7H4, wherein said second heavy chain consists of a heavy chain variable region as defined by SEQ ID NO: 29 and a heavy chain constant region as defined by SEQ ID NO: 61, and wherein said second light chain consists of a light chain variable region as defined by SEQ ID NO: 33 and a light chain constant region as defined by SEQ ID NO: 63.

In another further preferred embodiment, the pharmaceutical composition or unit dosage form comprises a bispecific antibody capable of binding human CD3 and human B7H4 is provided comprising:

- a first heavy chain and a first light chain which comprise the binding region capable of binding

to human CD3, wherein said first heavy chain consists of a heavy chain variable region as defined by SEQ ID NO: 17 and a heavy chain constant region as defined by SEQ ID NO: 60 with the terminal lysine (K) deleted, and wherein said first light chain consists of a light chain variable region as defined by SEQ ID NO: 22 and a light chain constant region as defined by SEQ ID NO: 64; and

a second heavy chain and a second light chain which comprise the binding region capable of binding to human B7H4, wherein said second heavy chain consists of a heavy chain variable region as defined by SEQ ID NO: 29 and a heavy chain constant region as defined by SEQ ID NO: 61 with the terminal lysine (K) deleted, and wherein said second light chain consists of a light chain variable region as defined by SEQ ID NO: 33 and a light chain constant region as defined by SEQ ID NO: 63.

Methods of preparing bispecific antibodies

Traditional methods such as the hybrid hybridoma and chemical conjugation methods (Marvin and Zhu (2005) Acta Pharmacol Sin 26:649) can be used in the preparation of the bispecific antibodies of the invention. Co-expression in a host cell of two antibodies, consisting of different heavy and light chains, leads to a mixture of possible antibody products in addition to the desired bispecific antibody, which can then be isolated by, e.g., affinity chromatography or similar methods.

Strategies favoring the formation of a functional bispecific, product, upon co-expression of different antibody constructs can also be used, e.g., the method described by Lindhofer et al. (1995 J Immunol 155:219). Fusion of rat and mouse hydridomas producing different antibodies leads to a limited number of heterodimeric proteins because of preferential species-restricted heavy/light chain pairing. Another strategy to promote formation of heterodimers over homodimers is a “knob-into-hole” strategy in which a protuberance is introduced on a first heavy-chain polypeptide and a corresponding cavity in a second heavy-chain polypeptide, such that the protuberance can be positioned in the cavity at the interface of these two heavy chains 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. 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 (US patent 5,731,168). EP1870459 (Chugai) and WO2009089004 (Amgen) describe other strategies for favoring heterodimer formation upon co-expression of different antibody domains in a host cell. In these methods, one or more residues that make up the CH3-CH3 interface in both CH3 domains are replaced with a charged amino acid such that homodimer formation is electrostatically unfavorable and heterodimerization is electrostatically favorable. WO2007110205 (Merck) describe yet another strategy, wherein differences between IgA

and IgG CH3 domains are exploited to promote heterodimerization.

Another *in vitro* method for producing bispecific antibodies has been described in WO2008119353 (Genmab), wherein a bispecific antibody is formed by "Fab-arm" or "half-molecule" exchange (swapping of a heavy chain and attached light chain) between two monospecific IgG4- or IgG4-like
5 antibodies upon incubation under reducing conditions. The resulting product is a bispecific antibody having two Fab arms which may comprise different sequences.

A preferred method for preparing the bispecific CD3xB7H4 antibodies of the present invention includes methods described in WO2011131746 and WO13060867 (Genmab) comprising the following steps:

10 a) providing a first antibody comprising an Fc region , said Fc region comprising a first CH3 region;

b) providing a second antibody comprising a second Fc region, said Fc region comprising a second CH3 region, wherein the first antibody is a CD3 antibody and the second antibody is a B7H4 antibody, or vice versa;

15 wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions;

c) incubating said first antibody together with said second antibody under reducing conditions; and

20 d) obtaining said bispecific CD3xB7H4 antibody.

In one embodiment, the said first antibody together with said second antibody are incubated under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerization, wherein the heterodimeric interaction between said first and second antibodies in the resulting heterodimeric antibody is such that no Fab-arm exchange occurs at 0.5 mM GSH after 24
25 hours at 37° C.

Without being limited to theory, in step c), the heavy-chain disulfide bonds in the hinge regions of the parent antibodies are reduced and the resulting cysteines are then able to form inter heavy-chain disulfide bond with cysteine residues of another parent antibody molecule (originally with a different specificity). In one embodiment of this method, the reducing conditions in step c) comprise the
30 addition of a reducing agent, e.g. a reducing agent selected from the group consisting of: 2-mercaptoethylamine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione, tris(2-carboxyethyl)phosphine (TCEP), L-cysteine and beta-mercapto-ethanol, preferably a reducing agent

selected from the group consisting of: 2-mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine. In a further embodiment, step c) comprises restoring the conditions to become non-reducing or less reducing, for example by removal of a reducing agent, e.g. by desalting.

For this method any of the CD3 and B7H4 antibodies described herein may be used. In a particular embodiment the CD3 and B7H4 antibodies, respectively, may be chosen so as to obtain a bispecific CD3xB7H4 antibody as described herein.

In one embodiment of this method, said first and/or second antibodies are full-length antibodies.

The Fc regions of the first and second antibodies may be of any isotype, including, but not limited to, IgG1, IgG2, IgG3 or IgG4. In one embodiment of this method, the Fc regions of both said first and said second antibodies are of the IgG1 isotype. In another embodiment, one of the Fc regions of said antibodies is of the IgG1 isotype and the other of the IgG4 isotype. In the latter embodiment, the resulting bispecific antibody comprises an Fc region of an IgG1 and an Fc region of IgG4 and may thus have interesting intermediate properties with respect to activation of effector functions.

In a further embodiment, one of the antibody starting proteins has been engineered to not bind Protein A, thus allowing to separate the heterodimeric protein from said homodimeric starting protein by passing the product over a protein A column.

As described above, the sequences of the first and second CH3 regions of the homodimeric starting antibodies are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions. More details on these interactions and how they can be achieved are provided in WO2011131746 and WO2013060867 (Genmab), which are hereby incorporated by reference in their entirety.

In particular, a stable bispecific CD3xB7H4 antibody can be obtained at high yield using the above method of the invention on the basis of two homodimeric starting antibodies which bind CD3 and B7H4, respectively, and contain only a few, fairly conservative, asymmetrical mutations in the CH3 regions. Asymmetrical mutations mean that the sequences of said first and second CH3 regions contain amino acid substitutions at non-identical positions.

In embodiment, a method for producing an antibody capable of binding to both B7H4 and CD3 in accordance with the invention is provided, comprising the steps of:

a) providing an antibody capable of binding to B7H4, said antibody comprising an antigen-binding region capable of binding to B7H4 as defined herein;
b) providing an antibody capable of binding to CD3, said antibody comprising an antigen-

binding region capable of binding to CD3 as defined herein;

c) incubating said antibody capable of binding to B7H4 together with said antibody capable of binding to CD3 under reducing conditions sufficient to allow cysteines in the hinge region to undergo disulfide-bond isomerization, and

5 d) obtaining said antibody capable of binding to B7H4 and CD3.

In such methods, the steps of providing an antibody capable of binding to B7H4 and/or CD3, may comprise the steps of

- providing cells containing expression vectors for producing said antibody or said antibodies; and

- allowing the cells to produce said antibody or said antibodies and subsequently,

10 - obtaining said antibody or said antibodies, thereby providing said antibody or said antibodies

Preferably, the antibody is comprised in a pharmaceutically acceptable carrier in the pharmaceutical compositions of the present invention. The pharmaceutical composition of the present invention may contain a bispecific antibody of the present invention targeting both B7H4 and CD3. The pharmaceutical composition may also comprise an antibody targeting B7H4. The pharmaceutical 15 composition may also comprise a combination of antibodies, including an antibody targeting B7H4 and/or a bispecific antibody in accordance with the present invention.

The pharmaceutical composition in accordance with the invention is preferably for use as a medicament. The pharmaceutical composition in accordance with the invention is preferably for use in the treatment of disease. Bispecific antibodies of the invention may be used for a number of 20 purposes. In particular, the bispecific antibodies of the invention may be used for the treatment of various forms of cancer, including metastatic cancer and refractory cancer. Preferably, the cancer may be of the solid tumor type.

In particular, the bispecific antibodies according to the invention may be useful in therapeutic settings in which specific targeting and T cell-mediated killing of cells that express B7H4 is desired.

25 In one embodiment, the present invention provides a method for treating a cancer in a subject, which method comprises administration of a therapeutically effective amount of a bispecific B7H4xCD3 antibody of the pharmaceutical composition of present invention. In a further embodiment, the present invention provides a method for treating a disorder involving cells expressing B7H4, in a subject, which method comprises administration of a therapeutically effective amount of a bispecific 30 antibody of the present invention.

In another embodiment, the present invention provides a method for treating a cancer in a subject, which method comprises administration of a therapeutically effective amount of an antibody capable

of binding to human B7H4 of the present invention. In a further embodiment, the present invention provides a method for treating a disorder involving cells expressing B7H4, in a subject, which method comprises administration of a therapeutically effective amount of a monospecific antibody of the present invention that is capable of binding to human B7H4.

5 As said, suitable diseases that can be contemplated in methods and uses in accordance with the invention are cancer. Said cancer most preferably is characterized by expression of B7H4. Expression of B7H4 in a cancer can easily be determined using methods known in the art, such as PCR, immunostaining, or FACS analysis, i.e. detecting expression of B7H4 transcript and/or protein. The antibodies as described herein that are capable of binding to human B7H4 may be used e.g. in
10 immunostaining and/or FACS analysis or the like.

Cancers that can express B7H4 include Breast cancer, Uterine/endometrial cancer, Uterine carcinosarcoma cancer, Ovarian cancer, Cervical cancer, Non-small cell lung cancer (squamous cell carcinoma and adenocarcinoma), Head and neck squamous cell carcinoma, Bladder cancer, esophageal cancer, cholangiocarcinoma, Pancreatic cancer, Stomach cancer, Renal cancer and
15 Prostate cancer.

Cancers that can express B7H4 include cancers such as cancers of the stomach, cholangiocarcinoma, bladder cancer, non small cell lung cancer (in particular squamous NSCLC), pancreatic cancer, cervical cancer, head and neck cancer, breast cancer (including triple negative breast cancer), ovarian cancer and uterine cancer. Types of cancers that may be preferred are cancers selected from uterine
20 carcinosarcoma (UCS), bladder urothelial carcinoma (BLCA), pancreatic adenocarcinoma (PAAD), lung squamous cell carcinoma (LUSC), breast invasive carcinoma (BRCA), uterine corpus endometrial carcinoma (UCEC), ovarian serous cystadenocarcinoma (OV) and cholangiocarcinoma (CHOL).

In a further embodiment, a patient being diagnosed with cancer may be subjected to an assessment of B7H4 expression in the cancer cells, and when B7H4 is detected, which may be in the range from
25 low to high, such a patient may be selected for treatment with an antibody in accordance with the invention. Patients diagnosed with having cancer of the stomach, cholangiocarcinoma, bladder cancer, non small cell lung cancer (in particular squamous NSCLC), pancreatic cancer, cervical cancer, head and neck cancer, breast cancer (including triple negative breast cancer), ovarian cancer or uterine cancer, may be subjected to such test. In a further embodiment, a patient being diagnosed
30 with having uterine carcinosarcoma (UCS), bladder urothelial carcinoma (BLCA), pancreatic adenocarcinoma (PAAD), lung squamous cell carcinoma (LUSC), breast invasive carcinoma (BRCA), uterine corpus endometrial carcinoma (UCEC), ovarian serous cystadenocarcinoma (OV) or cholangiocarcinoma (CHOL), may be subjected to such test. However, it may not necessarily be a

requirement to include such an assessment in selecting a patient for treatment.

Kits

The invention further provides a kit-of-parts comprising a pharmaceutical composition or a unit dosage form comprising an antibody as disclosed above, such as a kit for use as a companion diagnostic/for identifying within a population of patients, those patients which have a propensity to respond to treatment with an antibody as defined herein above or an immunoconjugate or antibody-drug conjugate (ADC) as defined herein above, or for predicting efficacy or anti-tumor activity of said antibody or immunoconjugate or ADC when used in treatment of a patient, the kit comprising an antibody as defined above; and instructions for use of said kit.

A kit-of-parts, such as a kit for use as a companion diagnostic/for identifying within a population of patients those patients which have a propensity to respond to treatment with an antibody as defined in any one of claims 1 to 55, comprising an antibody as defined in any one of claims 1 to 55; and instructions for use of said kit.

Hence, in one aspect, the invention relates to a diagnostic composition comprising a bispecific CD3xB7H4 antibody as defined herein, or a B7H4 antibody as defined herein, and to its use.

In another aspect, the invention relates to a kit for detecting cross-linking between CD3- and B7H4 expressing cells, in a sample derived from a patient, comprising

i) a bispecific antibody according to any one of the embodiments as disclosed herein; and

ii) instructions for use of said kit.

In one embodiment, the present invention provides a kit for diagnosis of cancer comprising a container comprising a bispecific CD3xB7H4 antibody, and one or more reagents for detecting cross-linking of B7H4 expressing cells and CD3 expressing cells. Reagents may include, for example, fluorescent tags, enzymatic tags, or other detectable tags. The reagents may also include secondary or tertiary antibodies or reagents for enzymatic reactions, wherein the enzymatic reactions produce a product that may be visualized.

In a further aspect, the invention relates to a method for detecting whether cross-linking between CD3- and B7H4-expressing cells occurs in a sample derived from a patient, upon administration of a bispecific antibody according to any one of the embodiments as disclosed herein, comprising the steps of:

(i) contacting the sample with a bispecific antibody according to any one of the embodiments as disclosed herein under conditions that allow for formation of a complex between

said bispecific antibody and the CD3-expressing cells and the B7H4- expressing cells; and

(ii) analyzing whether a complex has been formed.

Specific embodiments of the invention

5 1. A pharmaceutical composition comprising:

a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions are humanized and/or human, and

10 b) a buffering agent,

wherein the pH of the composition is from 4.0 to 8.0.

2. A pharmaceutical composition according to embodiment 1, wherein the pH of the composition is from 4.5 to 6.5.

15

3. A pharmaceutical composition according to embodiment 1, wherein the pH of the composition is from 5.0 to 6.0.

4. A pharmaceutical composition according to any one of the preceding embodiments, wherein the
20 buffering agent is selected from the group consisting of histidine, glutamate, and mixtures thereof.

5. A pharmaceutical composition according to any one of the preceding embodiments, wherein the pharmaceutical composition further comprises c) a non-ionic excipient.

25 6. A pharmaceutical composition according to embodiment 5, wherein the non-ionic excipient is a sugar or a sugar alcohol.

7. A pharmaceutical composition according to embodiments 5 or 6, wherein the non-ionic excipient is selected from sorbitol, sucrose or mixtures thereof.

8. A pharmaceutical composition according to any of embodiments 5-7, wherein the non-ionic excipient is present at a concentration of 100 to 300 mM, such as 125-250 mM preferably 250 mM.

9. A pharmaceutical composition according to any one of the preceding embodiments wherein the pharmaceutical composition further comprises d) a surfactant.

10. A pharmaceutical composition according to embodiment 9, wherein the surfactant is selected from the group consisting of glycerol monooleate, benzethonium chloride, sodium docusate, phospholipids, polyethylene alkyl ethers, sodium lauryl sulfate and tricaprylin, benzalkonium chloride, citrimide, cetylpyridinium chloride and phospholipids, alpha tocopherol, glycerol monooleate, myristyl alcohol, phospholipids, poloxamers, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbintan fatty acid esters, polyoxyethylene sterarates, polyoxyl hydroxystearate, polyoxylglycerides, polysorbates, propylene glycol dilaurate, propylene glycol monolaurate, sorbitan esters sucrose palmitate, sucrose stearate, tricaprylin and TPGS, and mixtures thereof.

11. A pharmaceutical composition according to embodiments 9 or 10, wherein the surfactant is a polysorbate.

12. A pharmaceutical composition according to embodiment 11, wherein the polysorbate is polysorbate 20 or 80, preferably polysorbate 80.

13. A pharmaceutical composition according to any of embodiments 9-12, wherein the surfactant is present at a concentration from about 0.005% to 0.4% w/v, such as from about 0.01 to 0.1 % w/v, such as from about 0.01 to 0.09 % w/v such as from about 0.01 to 0.06 % w/v such as from about 0.01 to 0.05% w/v such as 0.02% w/v or 0.03% w/v or 0.04% w/v or 0.05% w/v, preferably 0.02% w/v.

14. A pharmaceutical composition according to any one of the preceding embodiments, wherein the concentration of the antibody is 0.5 to 100 mg/ml, such as 1.0 to 50 mg/ml, or such as 5 to 30 mg/ml, such as 5 mg/ml, or 6 mg/ml, or 7 mg/ml, or 8 mg/ml, or 9 mg/ml, or 10 mg/ml, or 11 mg/ml, or 12 mg/ml, or 13 mg/ml, or 14 mg/ml, or 15 mg/ml, or 16 mg/ml, or 17 mg/ml, or 18 mg/ml, or 19 mg/ml,

or 20 mg/ml, or 21 mg/ml, or 22 mg/ml, or 23 mg/ml, or 24 mg/ml, or 25 mg/ml, or 26 mg/ml, or 27 mg/ml, or 28 mg/ml, or 29 mg/ml, 30 mg/ml, 31 mg/ml, 32 mg/ml, 33 mg/ml, 34 mg/ml, 35 mg/ml, 36 mg/ml, 37 mg/ml, 38 mg/ml, 39 mg/ml, 40 mg/ml, 41 mg/ml, 42 mg/ml, 43 mg/ml, 44 mg/ml, 45 mg/ml, 46 mg/ml, 47 mg/ml, 48 mg/ml, 49 mg/ml, 50 mg/ml, 51 mg/ml, 52 mg/ml, 53 mg/ml, 54 mg/ml, 55 mg/ml, 56 mg/ml, 57 mg/ml, 58 mg/ml, 59 mg/ml, or such as 60 mg/ml.

15. A pharmaceutical composition according to any one of the preceding embodiments, wherein the buffering agent is present at a concentration of 5 to 40 mM, such as 10-30 mM, preferably 20 mM.

16. A pharmaceutical composition according to any one of the preceding embodiments, wherein the pharmaceutical composition is an aqueous composition.

17. A pharmaceutical composition according to any one of the preceding embodiments, comprising:

a) 5-50 mg/ml of the antibody,

b) 10 to 20 mM glutamate or histidine,

c) 150 to 350 mM sorbitol or sucrose,

d) a polysorbate,

wherein the pH of the composition is from 5.0 to 6.0.

18. A pharmaceutical composition according to any one of the preceding embodiments, selected from the group consisting of

a pharmaceutical composition comprising a) 10-20 mg/ml of the antibody, b) 20 mM glutamate, c) 250 mM sorbitol, d) 0.02% w/v polysorbate 80, wherein the pH of the composition is from 5.1-5.3, and

a pharmaceutical composition comprising a) 10-20 mg/ml of the antibody, b) 20 mM histidine, c) 250 mM sucrose, d) 0.02% w/v polysorbate 80, wherein the pH of the composition is from 5.4-5.6.

19. A pharmaceutical composition according to any one of the preceding embodiments, wherein the

composition is a liquid composition.

20. A pharmaceutical composition according to any embodiments 1-19, wherein the composition is an intravenous composition, and/or wherein the composition is for use in intravenous administration.

5 21. A pharmaceutical composition according to any one of the preceding embodiments, wherein the composition is for use in the treatment of cancer.

22. The pharmaceutical composition of any of the preceding embodiments which is in a unit dosage form.

10

23. A pharmaceutical composition according to any one of the preceding embodiments, wherein the composition is stable for pharmaceutical use for at least 6 months, such as at least 9 month or at least 12 months at a storage temperature of 2-8°C, such as 5°C.

15 24. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is a bispecific antibody.

25. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is capable of binding cancer cells and T cells.

20

26. A pharmaceutical composition according to embodiment 25, wherein the cancer cells express said human B7H4.

27. A pharmaceutical composition according to embodiment 25 or 26, wherein the cancer cells are of

25 a solid tumor.

28. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is capable of inducing T cell mediated cell killing.

29. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding CD3, is capable of binding human CD3ε (epsilon), such as human CD3ε (epsilon) as specified in SEQ ID NO: 13.

5 30. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 16 or of SEQ ID NO. 17,

and,

10 a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22.

31. A pharmaceutical composition according to any of embodiments 1-29, wherein the antigen-binding region that binds to CD3 comprises

15 a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NOs.: 18, 19 and 20 or 18, 19 and 21 respectively;

and,

a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively.

20

32. A pharmaceutical composition according to any of embodiments 1-29, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 17, or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity

25 to the sequence of SEQ ID NO: 17;

and,

a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22 or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 22.

30

33. A pharmaceutical composition according to any one of the preceding embodiments, wherein the dissociation equilibrium constant K_D between the antigen-binding region that binds to CD3 and CD3 is within the range of 1 – 100 nM, such as within the range of 5 – 100 nM, within the range of 10 – 100 nM, within the range of 1 – 80 nM, within the range of 1 – 60 nM within the range of 1 – 40 nM, within

the range of 1 – 20 nM, within the range of 5 – 80 nM, within the range of 5 – 60 nM, within the range of 5 – 40 nM, within the range of 5 – 20 nM, within the range of 10 – 80 nM, within the range of 10 – 60 nM, within the range of 10 – 40 nM, or such as within the range of 10 – 20 nM.

5 34. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody has a lower binding affinity for human CD3ε than an antibody having an antigen-binding region comprising a VH sequence as set forth in SEQ ID NO: 16, and a VL sequence as set forth in SEQ ID NO: 22, preferably wherein said affinity is at least 5-fold lower, such as at least 10-fold lower, e.g. at least 20-fold lower, at least 30 fold lower, at least 40 fold lower, at least 45 fold lower or such as at
10 least 50-fold lower.

35. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region that binds to CD3 has an equilibrium dissociation constant K_D which is within the range of 200 – 1000 nM, such as within the range of 300 – 1000 nM, within the range of 400 –
15 1000 nM, within the range of 500 – 1000 nM, within the range of 300 – 900 nM within the range of 400 – 900 nM, within the range of 400 – 700 nM, within the range of 500 – 900 nM, within the range of 500 – 800 nM, within the range of 500 – 700 nM, within the range of 600 – 1000 nM, within the range of 600 – 900 nM, within the range of 600 – 800 nM, or such as within the range of 600 – 700 nM.

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36. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region that binds to CD3 comprises a heavy chain variable (VH) region comprising a CDR1 sequence, a CDR2 sequence and a CDR3 sequence,

the heavy chain variable (VH) region, when compared to a heavy chain variable (VH) region comprising
25 the sequence set forth in SEQ ID NO: 16 has an amino acid substitution being at a position selected from the group consisting of: T31, N57, H101, G105, S110 and Y114, the positions being numbered according to the sequence of SEQ ID NO: 16; and

the wild type light chain variable (VL) region comprises the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO: 23, GTN and SEQ ID NO: 24, respectively.

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37. A pharmaceutical composition according to embodiment 36, wherein the antigen-binding region that binds to CD3 comprises in the heavy chain variable (VH) region a substitution selected from the group consisting of: T31M, T31P, N57E, H101G, H101N, G105P, S110A, S110G, Y114M, Y114R, Y114V.

38. A pharmaceutical composition according to any of embodiments 30-32, wherein the CDR1, CDR2 and CDR3 of the heavy chain variable (VH) region of the antigen-binding region that binds to CD3 comprises, in total, at the most 1, 2, 3, 4 or 5 amino acid substitutions, when compared with the CDR1, CDR2 and CDR3 of the sequences of SEQ ID NO: 16, said amino acid substitutions comprising preferably amino acid substitutions as defined in embodiment 36 or embodiment 37.

39. A pharmaceutical composition according to any one of the preceding embodiments, wherein said human B7H4 is human B7H4 of SEQ ID NO. 1.

40. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding to human B7H4 is capable of binding to the extracellular domain of human B7H4.

41. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding to human B7H4 is capable of binding to the IgC-like constant region of human B7H4.

42. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding to human B7H4 is capable of binding to B7H3-IgV/B7H4-IgC having the sequence of SEQ ID NO. 11.

43. A pharmaceutical composition according to embodiment 42, wherein the antigen-binding region capable of binding to human B7H4 is not capable of binding to B7H4-IgV/B7H3-IgC having the sequence of SEQ ID NO. 10.

44. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding to human B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 36 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 40;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 43 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 47;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 50 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO.54; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 31 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 65 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 69.

45. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding to human B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 37, 38 and 39, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 41, DTS and SEQ ID NO. 42;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively

of SEQ ID NOs.: 44, 45 and 46, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 48, YTS and SEQ ID NO. 49;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 51, 52 and 53, and a variable light chain region comprising the CDR1, CDR2 and
5 CDR3 respectively of SEQ ID NO. 55, GAS and SEQ ID NO. 56; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 32 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively
10 of SEQ ID NOs.: 66, 67 and 68, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 70, GAS and SEQ ID NO. 71.

46. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen-binding region capable of binding to human B7H4 comprises:

15 a) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ ID NO. 33;

b) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33;

c) a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ
20 ID NO. 40;

d) a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47;

e) a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; or

25 f) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33

g) a variable heavy chain (VH) region of SEQ ID NO. 65 : and a variable light chain region of SEQ ID NO. 69.

30 47. A pharmaceutical composition according to any one of the preceding embodiments, wherein the

antigen-binding region capable of binding to human B7H4 has a binding affinity that corresponds to a K_D value of 5E-7 M or less, such as 1E-7 M or less, such as with a binding affinity corresponding to a K_D value which is within the range of 5E-7 to 2E-10 M, such as within the range of 2E-7 to 1E-10 M or 1E-7 to 5E-9 M.

5

48. A pharmaceutical composition according to embodiment 47, wherein the binding affinity is determined by biolayer interferometry, optionally as set forth in Example 3 herein.

49. A pharmaceutical composition according to any one of embodiments 47 and 48, wherein the
10 binding affinity is determined using a biolayer interferometry comprising the steps of:

I) immobilizing the antibody at an amount of 1 µg/mL for 600 seconds on an anti-human IgG Fc Capture biosensor;

II) determining association over a time period of 300 seconds and dissociation over a time period of 1000 seconds of human recombinant His tagged B7H4
15 protein (Sino Biological cat no 10738-H08H; a protein expressed from a construct of DNA sequence encoding the human VTCN1(Uniprot accession no. Q7Z7D3) (Phe29-Ala258) with a C-terminal polyhistidine tag) using a 2-fold dilution series ranging from 1.56 nM to 100 nM.

III) referencing the data to a buffer control (0 nM).

20

50. A pharmaceutical composition according to any one of embodiments 47 to 49, wherein the binding affinity is determined using an antibody as defined in any one of the preceding embodiments, which is a monospecific, bivalent antibody, such as an antibody which is a full length IgG1.

25 51. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen region capable of binding to human B7H4, said antigen-binding region capable of crossblocking

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 29 and a variable light chain region of SEQ ID NO. 33; and

30 an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40; and

wherein said antigen-binding region is not capable of crossblocking

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47;

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 65 and a variable light chain region of SEQ ID NO. 69.

52. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen region capable of binding to human B7H4, said antigen-binding region capable of crossblocking

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ ID NO. 47,

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 65 and a variable light chain region of SEQ ID NO. 69;

and wherein said antigen-binding region is not capable of crossblocking an antibody comprising

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 29 and a variable light chain region of SEQ ID NO. 33 and

an antibody comprising a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40.

53. A pharmaceutical composition according to any one of the preceding embodiments, wherein said antigen-binding region capable of binding to human B7H4 is capable of binding to B7H3-IgV/B7H4-IgC of SEQ ID NO. 11, and optionally is not capable of binding to B7H4-IgV/B7H3-IgC of SEQ ID NO. 10.

54. A pharmaceutical composition according to any one of the preceding embodiments, wherein each antigen-binding region comprises a heavy chain variable region (VH) and a light chain variable region (VL), and wherein said variable regions each comprise three CDR sequences, CDR1, CDR2 and CDR3,

respectively, and four framework sequences, FR1, FR2, FR3 and FR4, respectively.

55. A pharmaceutical composition according to any one of the preceding embodiments, wherein
the antigen-binding region capable of binding to B7H4 is human, and
5 the antigen-binding region capable of binding to CD3, is humanized.

56. A pharmaceutical composition according to any one of the preceding embodiments, wherein
the antigen-binding region capable of binding to B7H4 is human, and/or
the antigen-binding region capable of binding to CD3, is humanized.
10

57. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises two heavy chain constant regions (CH), and two light chain constant regions (CL).

58. A pharmaceutical composition according to embodiment 57, wherein the two heavy chain
15 constant domains, and two light chain constant regions are derived from human.

59. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is a full-length antibody.

20 60. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is of the IgG1 isotype.

61. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises a first and a second heavy chain, each of said first and second heavy chain
25 comprises at least a hinge region, a CH2 and CH3 region, wherein in said first heavy chain at least one of the amino acids in the positions corresponding to positions selected from the group consisting of T366, L368, K370, D399, F405, Y407 and K409 in a human IgG1 heavy chain has been substituted, and in said second heavy chain at least one of the amino acids in the positions corresponding to a position selected from the group consisting of T366, L368, K370, D399, F405, Y407, and K409 in a human IgG1
30 heavy chain has been substituted, wherein said substitutions of said first and said second heavy chains

are not in the same positions, and wherein the amino acid positions are numbered according to Eu numbering.

62. A pharmaceutical composition according to embodiment 61, wherein the amino acid in the position corresponding to K409 in a human IgG1 heavy chain is R in said first heavy chain, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain is L in said second heavy chain, or *vice versa*.

63. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises a first and, optionally, a second heavy chain and wherein the first heavy chain, and the second heavy chain if present, is/are modified so that the antibody induces Fc-mediated effector function to a lesser extent relative to an identical non-modified antibody.

64. A pharmaceutical composition according to embodiment 63, wherein the antibody comprises a first and a second heavy chain, and wherein in both the first and the second heavy chain, the amino acid residues at the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to Eu numbering are F and E, respectively.

65. A pharmaceutical composition according to embodiment 63 or embodiment 64, wherein the antibody comprises a first and a second heavy chain, and wherein in both the first and the second heavy chain, the amino acid residue at the position corresponding to position D265 in a human IgG1 heavy chain according to Eu numbering is A.

66. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises a kappa (κ) light chain.

67. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises a lambda (λ) light chain.

68. A pharmaceutical composition according to any one of the preceding embodiments, wherein the

antibody comprises a lambda (λ) light chain and a kappa (κ) light chain; e.g. an antibody with a heavy chain and a lambda light chain which comprise the binding region capable of binding to CD3, and a heavy chain and a kappa light chain which comprise the binding region capable of binding to B7H4.

5 69. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antigen binding region capable of binding to human B7H4 is comprised in an heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a kappa light chain constant region; and wherein said antigen binding region capable of binding to human CD3 is comprised in a heavy chain and a light chain, said
10 heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a lambda light chain constant region.

70. A pharmaceutical composition according to embodiment 69, wherein one IgG1 heavy chain constant region is as defined in SEQ ID NO. 60 and the other is as defined in SEQ ID NO. 61, and wherein
15 said kappa light chain constant region is as defined in SEQ ID NO. 63 and said lambda light chain constant region is as defined in SEQ ID NO. 64.

71. A pharmaceutical composition according to embodiment 70, wherein said IgG1 heavy chain constant regions as defined in SEQ ID NO. 60 and 61 have their terminal lysines deleted.

20

72. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is devoid of, or has reduced Fc-mediated effector function, and the antibody :

a) is capable of binding to B7H4-expressing human tumor cells such as described in Examples 9 and 10 herein,
25 b) mediating concentration-dependent cytotoxicity in B7H4-expressing human tumor cells when using e.g. PBMCs or T cells as effector cells when assayed as described in Examples 11 and 12 herein,
c) is capable of mediating concentration-dependent cytotoxicity in one or more human B7H4-expressing tumor cell lines selected from the group consisting of MCF-7, MDA-MB-
30 468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650 when using e.g. PBMCs or T cells as effector cells when assayed as described in Examples 11 and 12 herein,
d) is capable of activating T cells *in vitro* in the presence of B7H4-expressing human tumor

cells; e.g. when assayed as described in Example 13 herein,

e) is capable of activating T cells *in vitro* in the presence of one or more B7H4-expressing human tumor cell lines selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650; e.g. when assayed as described in Example 13 herein,

f) is capable of inducing cytotoxicity of B7H4-expressing human tumor cells; e.g. when assayed as described in Examples 11 and 12 herein, and/or

g) is capable of inducing T cell mediated cytotoxicity in one or more B7H4-expressing human tumor cell lines selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650; e.g. when assayed as described in Examples 11 and 12 herein.

73. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody has an IC50 in the range of 0.001-5 microgram/ml, wherein the IC50 is determined in an in vitro cytotoxicity assay comprising the steps of:

i) providing isolated peripheral blood mononuclear cells (PBMCs) or purified T cells from healthy human donor buffy coats,

ii) providing B7H4-expressing tumor cells;

iii) combining PBMCs or purified T cells and a human B7H4-expressing tumor cell line selected from the group consisting of MCF-7, MDA-MB-468, SK-BR3, NIH-OVCAR-3, HCC1954, and NCI-H1650, in a plurality of samples, wherein the ratio of the number of T-cells from said PBMCs, or purified T-cells,. to the selected tumor cell is 8:1;

iv) providing said antibody in a dilution series to said samples, ranging e.g. from 0.0128 ng/mL to 10,000 ng/mL and

v) incubating the samples, e.g. for 72 hours at 37°C; and subsequently,

vi) assessing the viability of the B7H4-expressing tumor cells,

vii) determining the percentage of viable cells for each dilution sample, and

viii) determining the IC50.

74. A pharmaceutical composition according to embodiment 73, wherein the IC50 is in the range of 0.001 -0.03 microgram/ml.

75. A pharmaceutical composition according to embodiment 73, wherein the IC50 is in the range of

0.05 – 5 microgram/ml.

76. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4, wherein said antigen-binding region capable of binding to human B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

e) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ ID NO. 33; or

f) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33.

77. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is a bivalent antibody.

78. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises a heavy chain variable region (VH), wherein CDR1 is according to SEQ ID NO. 18, wherein CDR2 is according to SEQ ID NO. 19, and wherein CDR3 is according to SEQ ID NO. 21.

79. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 23, wherein CDR2 is GTN, and wherein CDR3 is according to SEQ ID NO: 24.

80. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region capable of binding to B7H4 comprises a variable heavy chain (VH) region, wherein CDR1 is according to SEQ ID NO. 26, wherein CDR2 is according to SEQ ID NO. 30, and wherein CDR3 is according to SEQ ID NO. 28.

81. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region capable of binding to B7H4 comprises a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 34, wherein CDR2 is GAS, and wherein CDR3 is according to SEQ ID NO: 35.

82. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH), wherein ÇDR1 is according to SEQ ID NO. 18, wherein CDR2 is according to SEQ ID NO. 19, and wherein CDR3 is according to SEQ ID NO. 21, and

a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 23, wherein CDR2 is GTN, and wherein CDR3 is according to SEQ ID NO: 24, and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region, wherein ÇDR1 is according to SEQ ID NO. 26, wherein CDR2 is according to SEQ ID NO. 30, and wherein CDR3 is according to SEQ ID NO. 28, and

a light chain variable region (VL), wherein CDR1 is according to SEQ ID NO. 34, wherein CDR2 is GAS, and wherein CDR3 is according to SEQ ID NO: 35.

83. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

5 a heavy chain variable region (VH) comprising,the CDR1, CDR2, and CDR3 regions of SEQ ID NO. 17, and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22. and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID
10 NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33.

84. A pharmaceutical composition according to any one of the preceding embodiments, wherein the antibody is bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, or a biosimilar thereof.

15

85. The pharmaceutical composition as defined in any of embodiments 1 to 84 for use as a medicament.

86. The pharmaceutical composition for use as a medicament according to embodiment 85 for use in
20 a method for the treatment of a disease.

87. The pharmaceutical composition for use according to embodiment 86, wherein the disease is cancer.

25 88. The pharmaceutical composition for use according to embodiment 87, wherein the cancer is characterized by expression of B7H4 in cancer cells.

89. The pharmaceutical composition for use according to embodiment 88, wherein said expression of B7H4 is determined in cancer cells obtained from a patient.

30

90. The pharmaceutical composition for use according to embodiments 87-89, wherein the cancer is a solid tumor.

91. The pharmaceutical composition for use according to any one of embodiments 87 to 90, wherein the cancer is selected from the group consisting of lung cancer, NSCLC (ADC or SQCC), stomach cancer, pancreas cancer, cholangiocarcinoma, bladder cancer, cervical cancer, head and neck cancer, breast cancer, ovarian cancer and uterine cancer.

92. A method of treating a disease, the method comprising administering a pharmaceutical composition as defined in any one of embodiments 1-84 to a subject in need thereof.

93. The method according to embodiment 92, said method being for treatment of a cancer.

94. The method according to embodiment 93, wherein the cancer is selected from the group consisting of uterine carcinosarcoma (UCS), bladder urothelial carcinoma (BLCA), pancreatic adenocarcinoma (PAAD), lung squamous cell carcinoma (LUSC), breast invasive carcinoma (BRCA), uterine corpus endometrial carcinoma (UCEC), ovarian serous cystadenocarcinoma (OV) and cholangiocarcinoma (CHOL).

95. A method of treating cancer in a subject comprising administering to a subject in need thereof the pharmaceutical composition of any one of embodiments 1 to 84 for a time sufficient to treat the cancer.

96. The method of embodiment 95 wherein the composition is administered intravenously.

97. Use of the pharmaceutical composition of any one of embodiments 1-84 for the treatment of cancer.

98. Use of the pharmaceutical composition of any one of embodiments 1-84 or 97, wherein the use is for intravenous administration.

99. A unit dosage form, comprising

a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions
5 are humanized and/or human, in an amount of from 5 pg to 1200 mg,

and

b) a buffering agent, preferably selected from the group consisting of histidine, glutamate, and mixtures thereof,

wherein the pH of the unit dosage form is from 4.0 to 8.0, preferably 4.5 to 6.5, more preferably 5.0
10 to 6.0.

100. A unit dosage form of embodiment 99, wherein the antibody is as defined in any of embodiments 24-84.

15 101. A unit dosage form of embodiments 99 or 100, wherein the amount of the antibody is from 20 mg to 1000 mg.

102. A unit dosage form of any one of embodiments 99 to 101 wherein the amount of the antibody is from 40 mg to 1000 mg, such as 40 mg, 50 mg, 100 mg, 150 mg, 160 mg, 170 mg, 180 mg, 190 mg,
20 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, or such as 1 g.

103. A unit dosage form of any one of embodiments 99-102, wherein the total volume is from 20 ml to 200 ml, wherein the dosage form is for I.V. administration.

25 104. A method of treating cancer in a subject comprising administering to a subject in need thereof the unit dosage form of any one of embodiments 99 to 103 for a time sufficient to treat the cancer.

105. A unit dosage form of any one of embodiments 99 to 103 for use in the treatment of cancer.

106. A container comprising the unit dosage form of any one of embodiments 99-103 or the pharmaceutical composition of any one of embodiments 1-84.

107. A kit-of-parts comprising:

5 a. the pharmaceutical composition of any one of embodiments 1-84, or the unit dosage form of any of embodiments 99-103,

b. a receptacle for the pharmaceutical composition or for the unit dosage form

c. directions for dilution and/or for use.

108. A kit-of-parts comprising:

10 a. the pharmaceutical composition of any one of embodiments 1-84, or the unit dosage form of any of embodiments 99-103,

b. a diluent,

c. a receptacle for the unit dosage form, and

d. directions for dilution and/or for use.

15

109. A kit-of-parts, such as a kit for use as a companion diagnostic/for identifying within a population of patients those patients which have a propensity to respond to treatment with a pharmaceutical composition as defined in any one of embodiments 1-84, comprising a pharmaceutical composition as defined in any one of embodiments 1-84; and instructions for use of said kit.

20

110. A method of preparing a pharmaceutical composition as defined in any one of embodiments 1-84, comprising the steps of mixing in water for injection:

a. 0.5 to 120 mg/ml of the antibody, and

b. a buffering agent

25 and adjusting the pH to 4.0-8.0, preferably to 5.0-6.0.

111. A method of preparing a unit dosage form as defined in any one of embodiments 99-103, comprising the steps of:

a. preparing the pharmaceutical composition by the steps of the method of embodiment 110, or

providing a pharmaceutical composition as defined in any one of embodiments 1-84;

b. providing a diluent

c. mixing the pharmaceutical composition and the diluent to a desired antibody concentration.

5 112. A pharmaceutical composition or a unit dosage form, which is obtainable by any of the methods as defined in embodiments 110 or 111.

The present invention is further illustrated by the following examples, which should not be construed as limiting the scope of the invention.

Example 1 - Generation of B7H4 antibodies and screenings materials

Expression of B7H4 constructs

5 Constructs encoding various full length B7H4 variants were generated: human (*Homo sapiens*) B7H4 (Uniprot accession no. Q7Z7D3), cynomolgus monkey (*Macaca fascicularis*) B7H4 transcript 1 (Uniprot accession no. A0A2K5U6P5), dog (*Canis familiaris*) B7H4 (Uniprot accession no. F1P8R9), rabbit (*Oryctolagus cuniculus*) B7H4 (Uniprot accession no. G1TQE8), rat (*rattus norvegicus*) B7H4 (Uniprot accession no. Q501W4), mouse (*mus musculus*) B7H4 (Uniprot accession no. Q7TSP5), and pig (*sus*
10 *scrofa*) B7H4 (Uniprot accession no. F1SAY4) (see Table 1).

In addition, a construct for the extracellular domain (ECD of human B7H4 (aa 25-259 from Uniprot accession no. Q7Z7D3) fused to human IgG1 Fc domain with a C-terminal His tag and C tag (B7H4ECD-FcHisC) (SEQ ID NO: 12) was generated. In SEQ ID NO: 1, amino acid residues 1-24 are a signal peptide; hence the mature B7H4ECD-FcHisC protein corresponds to amino acid residues 25-259 of SEQ ID NO:
15 1.

Constructs contained suitable restriction sites for cloning and an optimal Kozak (GCCGCCACC) sequence (Kozak, M., Gene 1999;234(2):187-208). The full length and ECD of B7H4 constructs were cloned in pSB, a mammalian expression vector containing Sleeping Beauty inverted terminal repeats flanking an expression cassette consisting of a CMV promoter and HSV-TK polyA signal.

20 *Generation of HEK-293F cell lines transiently expressing full length B7H4 variants*

Freestyle™ 293-F (a HEK-293 subclone adapted to suspension growth and chemically defined Freestyle medium [HEK-293F]) cells were obtained from Invitrogen (cat. no. R790-07) and transfected with the constructs described supra, using 293fectin (Invitrogen, cat. no. 12347-019) according to the manufacturer's instructions.

25 *Purification of His-tagged B7H4*

B7H4ECD-FcHisC was expressed using the Expi293F expression platform (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. A14527) essentially as described by the manufacturer.

The His-tag enables purification with immobilized metal affinity chromatography Ni-NTA. The His-tagged protein binds strongly to the column material, while other proteins present in the culture
30 supernatant do not bind or bind weakly compared to the His-tagged proteins and elute in the flow-

through. The column was washed in order to remove weakly bound proteins. The strongly bound His-tagged proteins were then eluted with a buffer containing imidazole, which competes with the binding of His to Ni^{2+}. The eluent was removed by buffer exchange on a desalting column.

Immunization

5 OmniRat® animals (transgenic rats expressing a diversified repertoire of antibodies with fully human idiotypes; Ligand Pharmaceuticals Inc., San Diego, USA) were immunized by subcutaneous injections in the hocks of both hind legs (twice weekly for 7 weeks) with 50 µg B7H4ECD-FcHisC in PBS mixed with an equal volume of adjuvant (Sigma adjuvant system (Sigma-Aldrich, St. Louis, MO, USA, cat. no. S6322) or CFA, Complete Freund Adjuvant (1st injection) and IFA, Incomplete Freund Adjuvant (Sigma-10 Aldrich, St. Louis, MO, USA, cat. no. F5881/F5506) (subsequent injections), followed by a final boost s.c. injection of antigen in PBS without adjuvant.

Antibody generation

Lymph node cells from immunized animals were fused to mouse myeloma SP2.0 cells according to standard procedures 3 days after the final boost. RNA from hybridomas producing B7H4 specific 15 antibody was extracted and 5'-RACE-complementary DNA (cDNA) was prepared from 100 ng total RNA, using the SMART RACE cDNA Amplification kit (Clontech), according to the manufacturer's instructions. VH and VL coding regions were amplified by PCR and cloned directly, in frame, in the p33G1f, p33Kappa and p33Lambda expression vectors (pcDNA3.3 based vectors with codon optimized human IgG1m(f), Kappa and Lambda constant domains respectively), by ligation independent cloning 20 (Aslanidis, C. and P.J. de Jong, Nucleic Acids Res 1990;18(20): 6069-74). The variable domains from these expression vectors were sequenced and CDRs were annotated according to IMGT definitions (Lefranc MP. et al., Nucleic Acids Research, 27, 209-212, 1999 and Brochet X. Nucl. Acids Res. 36, W503-508 (2008)). Clones with a correct Open Reading Frame (ORF) were expressed and tested for binding to the antigen. After antigen specific screening assay was performed, the sequences of 25 variable regions of heavy and light chain were gene synthesized and cloned into an expression vector including a human IgG1 heavy chain containing the following amino acid mutations: L234F, L235E, D265A and K409R (FEAR) wherein the amino acid position number is according to Eu numbering (correspond to SEQ ID NO 60), and into expression vectors including human kappa or lambda light chain. For some of the antibodies, a variant with point mutation in the variable domains was generated 30 to remove a cysteine residue, which potentially could generate undesired disulphide bridge formation, or to replace an Asparagine to Serine or germline residue to remove a potential N-linked glycosylation site. For example, from the C1 heavy and light chain variable region sequences, a variant with an N52S substitution was made corresponding with a substitution in CDR2 (see TABLE 1, SEQ ID NOs. 25 and

29), and a further variant can have an N52Q substitution (SEQ ID NO. 31).

Antigen specific screening assay

The presence of B7H4 antibodies in sera of immunized animals, or hybridoma and transfectoma culture supernatant was determined in a homogeneous binding assay. Samples were analyzed for binding of antibodies to HEK-293F cells transiently transfected with the constructs made to express full length B7H4 variants expressing human B7H4, cynomolgus monkey B7H4 or murine B7H4, or HEK-293F wild-type cells (negative control). Samples were added to the cells to allow antibody binding to B7H4. Subsequently, antibody binding was detected using an appropriate fluorescent conjugate (AffiniPure Goat Anti-Rat IgG (H+L) Alexa Fluor® 647; Jackson ImmunoResearch, cat no. 112-605-143; AffiniPure Goat Anti-Human IgG Fc gamma-Alexa Fluor® 647; Jackson ImmunoResearch, cat no. 109-605-098). Cells (2.5 x 10^5 cells/ml) were mixed with goat anti-human AffiniPure Goat Anti-Human IgG Fc gamma-Alexa Fluor® 647 (0.2 µg/ml; Jackson ImmunoResearch Laboratories, 109-605-098) or AffiniPure Goat Anti-Rat IgG (H+L) Alexa Fluor® 647 (0.2 µg/ml; Jackson ImmunoResearch, 112-605-143) depending on the backbone of the antibody. Serial dilutions of test and control antibodies (range 0.003 to 3 µg/mL in 2-fold dilution steps) were prepared and 2 µl antibody dilution was added to 5 µl of the cell/conjugate mixture in 1536 well plates (Greiner, cat. no. 789866). Plates were incubated at room temperature for 9 hours, and after which fluorescence intensity was determined using an ImageXpress Velos Laser Scanning Cytometer (Molecular Devices, LLC, Sunnyvale, CA, USA) and total fluorescence was used as read-out. Samples were stated positive when counts were higher than 50 and counts x fluorescence was at least three times higher than the negative control.

Results from B7H4 antibody panel generation

From 176 out of 193 hybridomas produced, heavy and light chain variable region sequences were successfully obtained. Of 351 heavy chain/light chain combinations tested, 98 showed binding in antigen screenings assays using human B7H4-transfected HEK-293F cells as described above. 35 antibodies were selected: 26 with original sequences and 9 variants with point mutations introduced in the variable domains. Antibodies were produced as monovalent binding antibodies (as CD3 bispecifics) and bivalent binding antibodies (as IgG1 molecules), and tested for binding to tumor cells as described below. Of the antibodies from the panel generated, only antibody B7H4-C1 and its variant B7H4-C1-N52S, of which the corresponding VH and VL antibody variable domain encoding sequences are listed in TABLE 1, provided for antibodies that bound to tumor cells as described below.

Further B7H4 antibodies

In the examples, further antibodies specific for B7H4 were used containing the variable domains

previously described in WO2014159835 (referenced therein as SEQ ID NOs 38 and 35), corresponding herein to B7H4-C2, relevant sequences of the variable domains are listed herein in TABLE 1 and include SEQ ID NO. 43 and 47; WO2014159835 (referenced therein as SEQ ID NO 56 and 55), corresponding herein to B7H4-C3, relevant sequences of the variable domains are listed herein in TABLE 1 and include
5 SEQ ID NO. 36 and 40; WO2009073533 (referenced therein as SEQ ID No 2 and 7), corresponding herein to B7H4-C4 and relevant sequences of the variable domains are listed herein in TABLE 1 and include SEQ ID NO. 50 and 54; and US20190085080A1 corresponding herein to B7H4-C5 and relevant sequences of the variable domains are listed herein in TABLE 1 and include SEQ ID NO. 65 and 69. The corresponding VH and VL antibody variable domain encoding sequences were synthesized and cloned
10 into pcDNA3.3 based vectors with codon optimized human IgG1m(f) and Kappa or Lambda constant domains, or variants thereof, to produce monospecific and bispecific antibodies. When reference is made to antibody IgG1-B7H4-CX -FEAL, this represents an antibody having the B7H4-CX variable regions, being of the IgG1 isotype, and having amino acid substitutions L234F, L235E, D265A and F409R in the constant region of the heavy chain.

15 *IgG1-b12 antibody*

The antibody b12, an HIV-1 gp120 specific antibody (Barbas, CF. J Mol Biol. 1993 Apr 5; 230(3):812-23) was used in some examples as a negative control IgG1, or as the non-binding control Fab-arm of a control bispecific. The codon optimized antibody encoding sequences for this control antibody were synthesized and cloned into pcDNA3.3 based vectors with codon optimized human IgG1m(f) and
20 Kappa constant domains, or variants thereof. The sequence of the variable heavy chain (VH) region and the sequence of the variable light chain (VL) region are included herein as SEQ ID NOs.: 14 and 15, respectively.

Example 2 - Humanized CD3 antibodies for the generation of CD3xB7H4 bispecific antibodies

The generation of humanized antibody IgG1-huCD3-H1L1 (of which the variable heavy and light chain
25 region sequences are listed herein in SEQ ID NO: 16 and 22) is described in Example 1 of WO2015/001085. IgG1-huCD3-H1L1 is referred to herein as 'IgG1-huCD3'. Antibody IgG1-huCD3-H1L1-FEAL is a variant hereof with three amino acid substitutions in the Fc domain (L234F, L235E, D265A), in addition to an amino acid substitution that allows the generation of bispecific antibodies through controlled Fab-arm exchange (F405L), as described herein below. It has been shown that such
30 mutations did not have effect on target binding of the antibodies in which they are introduced (see e.g. US 2015/0337049 and Engelberts et al., 2020, EBioMedicine 52: 102625).

The generation of humanized antibody IgG1-huCD3-H1L1-H101G (of which the variable heavy chain

and light chain region sequences are listed as SEQ ID NO: 17 and 22 herein) is described in Example 2 of WO2017/009442. IgG1-huCD3-H1L1-H101G will be referred to as 'IgG1-huCD3-H101G'. This variant comprises a substitution H101G in the variable heavy chain region sequence (compare SEQ ID NO.16 and 17), and has the same light chain as IgG1-huCD3-H1L1. Antibody IgG1-huCD3-H101G-FEAL is a
5 variant hereof with amino acid substitutions L234F, L235E, D265A and F405L.

Example 3 – B7H4 binding affinity determination using biolayer interferometry

Target binding affinity of B7H4 antibodies was determined by label-free biolayer interferometry (BLI) on an Octet HTX instrument (FortéBio). Experiments were carried out while shaking at 1,000 RPM at 30°C. Initially, the affinity of IgG1-B7H4-C1-N52S-FEAR, IgG1-B7H4-C2-FEAR, IgG1-B7H4-C3-FEAR, and
10 IgG1-B7H4-C4-FEAR for human and mouse B7H4 was determined using BLI. Anti-Human IgG Fc Capture (AHC) biosensors (FortéBio, cat. no. 18-5060) were pre-conditioned by exposure to 10 mM glycine (Sigma-Aldrich, cat. no. 15527) buffer pH 1.7 for 5 s, followed by neutralization in Sample Diluent (FortéBio, cat. no. 18-1048) for 5 s; both steps were repeated 2 times. Next, AHC sensors were loaded with antibody (1 µg/mL in Sample diluent) for 600 s. After a baseline measurement in Sample
15 Diluent (100 s), the association (300 s) and dissociation (1,000 s) of human B7H4 (Sino Biological, cat. no. 10738-H08H-100) or mouse B7H4 (R&D Systems, cat. no. 2154-B7-050) was determined using a concentration range of 1.56 - 100 nM (0.04 – 2.68 µg/mL) and 5.9 – 375 nM (0.16 – 10 µg/mL) for human and mouse B7H4 respectively, with two-fold dilution steps in Sample Diluent. The theoretical molecular mass of human B7H4 and mouse B7H4 (as ECD-His tagged molecules) based on their amino
20 acid sequences (26.8 kDa and 26.6 kDa respectively) were used for calculations. For each antibody a reference sensor was used, which was incubated with Sample Diluent instead of antigen. AHC sensors were regenerated by exposure to 10 mM glycine buffer pH 1.7 for 5 s, followed by neutralization in Sample Diluent for 5 s; both steps were repeated twice. Subsequently sensors were loaded again with antibody for the next cycle of kinetics measurements.

25 Data were acquired using Data Acquisition Software v9.0.0.49d (FortéBio) and analyzed with Data Analysis Software v9.0.0.12 (FortéBio). Data traces were corrected per antibody by subtraction of the reference sensor. The Y-axis was aligned to the last 10 s of the baseline, Interstep Correction alignment to dissociation and Savitzky-Golay filtering were applied. Data traces with a response < 0.05 nm were excluded from analysis. The data was fitted with the 1:1 Global Full fit model using a window of
30 interest for the association and dissociation times set at 300 s and 200 s respectively.

In a second experiment, the affinity of IgG1-B7H4-C1-N52S-FEAR, IgG1-B7H4-C2-FEAR, IgG1-B7H4-C3-FEAR, IgG1-B7H4-C4-FEAR, and IgG1-B7H4-C5-FEAR for human and mouse B7H4 was determined using BLI. The experiment was performed as described above, with some small exceptions. The

preconditioning steps were repeated 5 times. The association (200 s) and dissociation (1,000 s) of human or mouse B7H4 were determined using a concentration range of 0.78 – 800 nM with two-fold dilution steps in Sample Diluent. Data were acquired using Data Acquisition Software v12.0.1.8 (FortéBio) and analyzed with Data Analysis Software v12.0.1.2 (FortéBio). The data was fitted with the 1:1 Global Full Fit model using a window of interest for the association time of 200 s and a window of interest for the dissociation time of 200 s, except for IgG1-B7H4-C2-FEAR for which a 1,000 s dissociation time was used. The dissociation time was chosen based upon R^2 value, visual inspection of the curve and at least 5% signal decay during the dissociation step. Data traces generated with antigen concentrations higher than 100 nM were excluded from analysis for antibodies with an affinity below 50 nM.

In addition, the affinity of for cynomolgus monkey B7H4 was determined by BLI. In a first experiment, the affinity of bsIgG1-huCD3-FEALxB7H4-C1-FEAR, bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR, bsIgG1-huCD3-FEALxB7H4-C2-FEAR, bsIgG1-huCD3-FEALxB7H4-C3-FEAR, and bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR for cynomolgus monkey B7H4 was determined. Amine Reactive 2nd Generation (AR2G) biosensors (FortéBio, cat. no. 18-5092) were activated by reaction with 20 mM EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) (FortéBio, cat. no. 18-1033) and 10 mM s-NHS (N-hydroxysulfosuccinimide sodium salt) (FortéBio, cat. no. 18-1067) for 300 s. The activated sensors were loaded with 10 µg/mL recombinant hIgG1 Fc-tagged cynomolgus monkey B7H4 (Creative BioMart, cat. no. VTCN1-1517R) in 10 mM Sodium Acetate pH 4.0 (FortéBio, cat. no. 18-1068) for 600 s and quenched with 1 M ethanolamine pH 8.5 (FortéBio, cat. no. 18-1071) for 300 s. After a baseline measurement in Sample Diluent (300 s; FortéBio, cat. no. 18-1048), the association (100 s) and dissociation (1,000 s) of functionally monovalent B7H4 binding by CD3xB7H4 bispecific antibodies (as indicated in Table 8) was determined using a concentration range of 0.23 – 15 µg/mL (1.56 - 100 nM) with two-fold dilution steps in Sample Diluent. A molecular mass of 150 kDa of the antibodies was used for calculations. For each antibody a reference sensor was used, which was incubated with Sample Diluent instead of antibody.

Data were acquired using Data Acquisition Software v9.0.0.49d (FortéBio) and analyzed with Data Analysis Software v9.0.0.12 (FortéBio). Data traces were corrected per antibody by subtraction of the reference sensor. The Y-axis was aligned to the last 10 s of the baseline, Interstep Correction alignment to dissociation and Savitzky-Golay filtering were applied. Data traces with a response < 0.05 nm were excluded from analysis. The data was fitted with the 1:1 Global Full fit model using a window of interest for the association and dissociation times set at 100 s and 200 s respectively.

In a second experiment to determine the affinity of the B7H4 antibodies for cynomolgus monkey B7H4, the affinity of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-

FEALxB7H4-C5-FEAR was determined. The experiment was performed as described above, with some small exceptions. After a baseline measurement in Sample Diluent of 600 s, the association (200 s) and dissociation (1,000 s) of functionally monovalent B7H4 binding by CD3xB7H4 bispecific antibodies (as indicated in Table 9) was determined using a concentration range of approximately 0.1 – 116 µg/mL
5 (0.78 - 800 nM) with two-fold dilution steps in Sample Diluent. The specific molecular mass of each antibody (approximately 145 kDa) was used for calculations. Data were acquired using Data Acquisition Software v12 (FortéBio) and analyzed with Data Analysis Software v12 (FortéBio). Data traces with a response < 0.03 nm were excluded from analysis. The data was fitted with the 1:1 Global Full fit model using a window of interest for the association time and dissociation time of 200 s. The
10 dissociation time was chosen based upon R^2 value, visual inspection of the curve and at least 5% signal decay during the dissociation step. Data traces generated with antibody concentrations higher than 200 nM were excluded from analysis for antibodies with an affinity below 50 nM. All results were determined with an R^2 of at least 0.98.

"K_D" (M) refers to the equilibrium dissociation constant of the antibody-antigen interaction, and is
15 obtained by dividing k_d by k_a. "k_d" (sec^{-1}) refers to the dissociation rate constant of the antibody-antigen interaction. This is sometimes also referred to as the k_{off} value or off-rate. "k_a" (M^{-1} x sec^{-1}) refers to the association rate constant of the antibody-antigen interaction. This is sometimes also referred to as the k_{on} value or on-rate.

Tables 4 and 5 show the results of the first and the second experiment in which the association rate
20 constant k_a (1/Ms), dissociation rate constant k_d (1/s) and equilibrium dissociation constant K_D (M) of the indicated antibodies for human B7H4 were determined by biolayer interferometry.

Table 4. Binding affinities of antibodies to human B7H4 extracellular domain as determined by label-free biolayer interferometry. ND = not determined.

Antibody	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (M)
IgG1-B7H4-C1-FEAR	ND	ND	ND
IgG1-B7H4-C1-N52S-FEAR	9.4E+04	5.4E-03	5.7E-08
IgG1-B7H4-C2-FEAR	5.2E+04	8.8E-04	1.7E-08
IgG1-B7H4-C3-FEAR	9.9E+04	4.1E-03	4.2E-08
IgG1-B7H4-C4-FEAR	1.5E+05	1.6E-03	1.1E-08

Table 5. Binding affinities of antibodies to human B7H4 extracellular domain as determined by label-free biolayer interferometry.

Antibody	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (M)
IgG1-B7H4-C1-N52S-FEAR[1]	8.4E+04	4.7E-03	5.7E-08
IgG1-B7H4-C2-FEAR	5.9E+04	1.7E-04	3.0E-09
IgG1-B7H4-C3-FEAR	8.1E+04	4.4E-03	5.4E-08
IgG1-B7H4-C4-FEAR	2.2E+05	1.7E-03	7.9E-09
IgG1-B7H4-C5-FEAR	2.5E+05	2.5E-03	9.9E-09

[1] Shown are the averaged results of n=3 experiments.

5 Tables 6 and 7 show the results of two experiments in which the k_a (1/Ms), k_d (1/s), and K_D (M) of the indicated antibodies for mouse B7H4 were determined by biolayer interferometry.

Table 6. Binding affinities of antibodies to mouse B7H4 extracellular domain as determined by label-free biolayer interferometry. ND = not determined; - = no binding (response <0.05 nm at the highest concentration used).

Antibody	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (M)
IgG1-B7H4-C1-FEAR	ND	ND	ND
IgG1-B7H4-C1-N52S-FEAR	-	-	-
IgG1-B7H4-C2-FEAR	3.3E+04	7.7E-04	2.4E-08
IgG1-B7H4-C3-FEAR	5.1E+04	2.0E-02	3.9E-07
IgG1-B7H4-C4-FEAR	8.4E+04	1.4E-03	1.6E-08

10

Table 7. Binding affinities of antibodies to mouse B7H4 extracellular domain as determined by label-free biolayer interferometry. - = no binding (response <0.05 nm at the highest concentration used).

Antibody	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (M)

IgG1-B7H4-C1-N52S-FEAR	-	-	-
IgG1-B7H4-C2-FEAR	6.3E+04	1.3E-04	2.1E-09
IgG1-B7H4-C3-FEAR	5.9E+04	1.8E-02	3.0E-07
IgG1-B7H4-C4-FEAR	1.4E+05	1.4E-03	9.7E-09
IgG1-B7H4-C5-FEAR	1.7E+05	2.4E-03	1.4E-08

Tables 8 and 9 show the results of two experiments in which the k_a (1/Ms), k_d (1/s), and K_D (M) of the indicated antibodies for cynomolgus monkey B7H4 were determined by biolayer interferometry.

Table 8. Binding affinities of functionally monovalent antibodies to cynomolgus monkey B7H4
5 extracellular domain as determined by label-free biolayer interferometry.

Antibody	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (M)
bsIgG1-huCD3-FEALxB7H4-C1-FEAR	2.7E+05	1.4E-03	5.1E-09
bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR	1.4E+05	3.0E-03	2.1E-08
bsIgG1-huCD3-FEALxB7H4-C2-FEAR	1.3E+05	4.1E-04	3.1E-09
bsIgG1-huCD3-FEALxB7H4-C3-FEAR	2.8E+05	4.1E-03	1.5E-08
bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR	3.5E+05	1.5E-03	4.2E-09

Table 9. Binding affinities of functionally monovalent antibodies to cynomolgus monkey B7H4 extracellular domain as determined by label-free biolayer interferometry.

Antibody	R^2	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (M)
bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR[a]	0.99	1.2E+05	2.7E-03	2.5E-08
bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR[b]	0.97	4.2E+05	2.5E-03	6.0E-09

[a] Shown are the averaged results of n=3 experiments.
10 [b] Did not meet a stringent quality control R^2 threshold of 0.98.

Example 4 - CD3 binding affinity determination using biolayer interferometry

Binding affinities of IgG1-huCD3-FEAL and IgG1-huCD3-H101G-FEAL were determined as described in

Example 7 of WO2017/009442.

In short, binding affinities of selected CD3 antibodies in an IgG1-huCD3-FEAL format for recombinant soluble CD3ε (CD3E27-GSKa) (mature protein of SEQ ID NO: 13) were determined using biolayer interferometry on a ForteBio Octet HTX (ForteBio). Anti-human Fc capture biosensors (ForteBio, cat. no. 18-5060) were loaded for 600 s with hIgG (1 µg/mL). After a baseline measurement (200 s), the association (1000 s) and dissociation (2000 s) of CD3E27-GSKa was determined, using a CD3E27-GSKa concentration range of 27.11 µg/mL - 0.04 µg/mL (1000 nM - 1.4 nM) with three-fold dilution steps (sample diluent, ForteBio, cat. no. 18-5028). For calculations, the theoretical molecular mass of CD3E27-GSKa based on the amino acid sequence was used, i.e. 27.11 kDa. Experiments were carried out while shaking at 1000 rpm and at 30°C. Each antibody was tested in at least two independent experiments. Data was analyzed with ForteBio Data Analysis Software v8.1, using the 1:1 model and a global full fit with 1000 s association time and 100 s dissociation time. Data traces were corrected by subtraction of a reference curve (antibody on biosensor, measurement with sample diluent only), the Y-axis was aligned to the last 10 s of the baseline, and interstep correction as well as Savitzky-Golay filtering was applied. Data traces with a response <0.05 nm were excluded from analysis.

Table 10 shows the association rate constant k_a (1/Ms), dissociation rate constant k_d (1/s) and equilibrium dissociation constant K_D (M) for recombinant CD3ε determined by biolayer interferometry. IgG1-huCD3-FEAL showed a relatively high (K_D: 15 nM) binding affinity to recombinant CD3ε compared to IgG1-huCD3-H101G-FEAL (K_D: 683 nM).

Table 10: Binding affinities of monospecific, bivalent CD3 antibodies to recombinant CD3ε as determined by label-free biolayer interferometry

Antibody	On-rate k_a (1/Ms)	Off-rate k_d (1/s)	K_D (nM)
IgG1-huCD3-FEAL	2.7E+05	4.0E-03	15
IgG1-huCD3-H101G-FEAL	3.0E+04	2.0E-02	683

Example 5 - Cross-block of B7H4 antibodies determined by biolayer interferometry

Antibody cross-block analysis (epitope binning) in classical Sandwich format was performed by BLI on an Octet HTX instrument (ForteBio). A first cross-block experiment with IgG1-B7H4-C1-N52S-FEAR, IgG1-B7H4-C2-FEAR, IgG1-B7H4-C3-FEAR, and IgG1-B7H4-C4-FEAR was carried out while shaking at 1,000 RPM and at 30°C.

Amine Reactive 2nd Generation (AR2G) biosensors (FortéBio, cat. no. 18-5092) were activated for 300

s with a solution of 20 mM EDC (*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride) (Sigma-Aldrich, cat. no. 03449) and 10 mM s-NHS (*N*-Hydroxysulfosuccinimide sodium salt) (Sigma-Aldrich, cat. no. 56485). The activated AR2G sensors were loaded with 20 μg/mL first antibody in 10 mM Sodium Acetate pH 6.0 (FortéBio, cat. no. 18-1070) for 600 s and quenched with 1 M
5 ethanolamine pH 8.5 (FortéBio cat. no. 18-1071) for 300 s. After a baseline measurement in Sample Diluent (50 s; FortéBio, cat. no. 18-1048), the AR2G biosensors containing immobilized antibodies were loaded for 300 s with human B7H4 (100 nM or 2.68 μg/mL diluted in Sample Diluent; Sino Biological, cat. no 10738-H08H). The theoretical molecular mass of human B7H4 based on the amino acid sequence (26.8 kDa) was used for calculations. The association (300 s) of a second antibody (10
10 μg/mL in Sample Diluent) was determined. Sensors were regenerated by exposure to 10 mM glycine (Riedel-de Haën, cat. no. 15527) buffer pH 2.5 for 5 s, followed by neutralization in Sample Diluent for 5 s; both steps were repeated twice. Subsequently the sensors containing immobilized first antibody were used again, starting with the baseline step.

Data were acquired using Data Acquisition Software v9.0.0.49d (FortéBio) and analyzed with Data
15 Analysis HT Software v10.0.17 (FortéBio). Data traces were corrected by subtraction of a reference curve (Sample Diluent instead of second antibody) in order to correct for the dissociation of B7H4 from the immobilized first antibody. The Y-axis was aligned to the start of the association step and Savitzky-Golay filtering was applied. The corrected association responses of the second antibodies were plotted in a matrix format. In general, responses > 0.05 nm were considered non-cross-blocking
20 antibodies, while responses < 0.05 nm were considered to be blocking antibody pairs.

The cross-block experiment was repeated to also include IgG1-B7H4-C5-FEAR and was performed as described above, with minor adaptations. The experiment was carried out while shaking at 1,000 RPM and at 22°C. Data were acquired using Data Acquisition Software v12.0.1.8 (ForteBio) and analyzed with Data Analysis HT Software v12.0.1.55 (ForteBio). In general, responses > 0.1 nm were considered
25 non-cross-blocking antibodies, while responses < 0.1 nm were considered to be blocking antibody pairs.

Initial cross-block experiments were performed for antibodies IgG1-B7H4-C1-N52S-FEAR, IgG1-B7H4-C3-FEAR, IgG1-B7H4-C4-FEAR and IgG1-B7H4-C2-FEAR. The results are summarized in Table 11. A second set of cross-block experiments was performed to also include IgG1-B7H4-C5-FEAR. These
30 results are summarized in Table 12. The first column shows the immobilized antibodies; the first row shows the antibodies in solution (referred to as 'the second antibodies' above). Corrected association responses of the antibodies in solution are shown. Cross-block of antibodies is indicated by the dark grey color, and non-blocking antibody combinations are unmarked (transparent background), showing

that IgG1-B7H4-C1-N52S-FEAR,IgG1-B7H4-C3-FEAR, and IgG1-B7H4-C5-FEAR are cross-blocking with each other and not with IgG1-B7H4-C4-FEAR and IgG1-B7H4-C2-FEAR, and vice versa.

Table 11: First antibody cross-block experiment using biolayer interferometry.

The first column shows the immobilized antibodies and the first row shows the antibodies in solution. Corrected association responses of the antibodies in solution are shown. Cross-block of antibodies is indicated by the dark grey color, non-blocking antibody combinations are unmarked (transparent background).

Antibody cross-block	IgG1-B7H4-C1-N52S-FEAR	IgG1-B7H4-C3-FEAR	IgG1-B7H4-C4-FEAR	IgG1-B7H4-C2-FEAR
IgG1-B7H4-C1-N52S-FEAR	-0.01	0.00	0.80	0.56
IgG1-B7H4-C3-FEAR	-0.02	-0.01	0.97	0.53
IgG1-B7H4-C4-FEAR	0.82	0.56	-0.02	-0.01
IgG1-B7H4-C2-FEAR	0.74	0.54	0.00	0.00

Table 12: Second antibody cross-block experiment using biolayer interferometry.

The first column shows the immobilized antibodies and the first row shows the antibodies in solution. Corrected association responses of the antibodies in solution are shown. Cross-block of antibodies is indicated by the dark grey color, non-blocking antibody combinations are unmarked (transparent background).

Antibody cross-block	IgG1-B7H4-C1-N52S-FEAR	IgG1-B7H4-C3-FEAR	IgG1-B7H4-C2-FEAR	IgG1-B7H4-C4-FEAR	IgG1-B7H4-C5-FEAR
IgG1-B7H4-C1-N52S-FEAR	0	0.01	0.38	0.42	0.43

IgG1-B7H4-C3-FEAR	0.01	0	0.44	0.57	0.61
IgG1-B7H4-C2-FEAR	0.28	0.25	0.01	0.01	0.02
IgG1-B7H4-C4-FEAR	0.5	0.39	0	-0.01	0
IgG1-B7H4-C5-FEAR	0.67	0.57	-0.03	-0.03	-0.01

Example 6 - Generation of bispecific antibodies by 2-MEA-induced Fab-arm exchange

Bispecific antibodies were generated *in vitro* using the DuoBody® platform technology, i.e. 2-MEA-induced Fab-arm exchange as described in WO2011147986, WO2011131746 and WO2013060867 (Genmab) and Labrijn et al. (Labrijn et al., PNAS 2013, 110: 5145-50; Gramer et al., MAbs 2013, 5: 962-973). To enable the production of bispecific antibodies by this method, IgG1 molecules carrying specific point mutations in the CH3 domain were generated: in one parental IgG1 antibody the F405L mutation (i.e. the CD3 antibodies in this application), in the other parental IgG1 antibody the K409R mutation (i.e. the B7H4 or control, HIV-1 gp120-specific, antibodies in this application). In addition to these mutations, the parental IgG1 antibodies included substitutions L234F, L235E, D265A (FEA).

To generate bispecific antibodies, the two parental antibodies were mixed in equal mass amounts in PBS buffer (Phosphate Buffered Saline; 8.7 mM HPO_4^{2-}, 1.8 mM $H_2PO_4^-$, 163.9 mM Na^+, 140.3 mM Cl^-, pH 7.4). 2-mercaptoethylamine-HCl (2-MEA) was added to a final concentration of 75 mM and the reaction mixture was incubated at 31°C for 5 h. The 2-MEA was removed by dialysis into PBS buffer using 10 kDa molecular-weight cutoff Slide-A-Lyzer carriages (Thermo Fisher Scientific) according to the manufacturer's protocol in order to allow re-oxidation of the inter-chain disulfide bonds and formation of intact bispecific antibodies.

The following antibodies were used in the examples:

B7H4 antibodies

IgG1-B7H4-C1-FEAR (having the VH and VL sequences set forth in SEQ ID NO: 25 and SEQ ID NO: 33).

IgG1-B7H4-C1-N52S-FEAR (having the VH and VL sequences set forth in SEQ ID NO: 29 and SEQ ID NO: 33).

IgG1-B7H4-C2-FEAR having the VH and VL sequences set forth in SEQ ID NO: 43 and SEQ ID NO: 47).

IgG1-B7H4-C3-FEAR having the VH and VL sequences set forth in SEQ ID NO: 36 and SEQ ID NO: 40).

IgG1-B7H4-C4-FEAR having the VH and VL sequences set forth in SEQ ID NO: 50 and SEQ ID NO: 54).

IgG1-B7H4-C5-FEAR having the VH and VL sequences set forth in SEQ ID NO: 65 and SEQ ID NO: 69).

The annotation IgG1 indicates that full length antibodies of the IgG1 isotype were made, and the FEAR annotation indicates that the heavy chain constant regions contains amino acid substitutions L234F, L235E, D265A and K409R and the light chain constant regions were of the kappa type (SEQ ID NO. 61 and 63, respectively).

5 *CD3 antibodies*

IgG1-huCD3-FEAL (having the VH and VL sequences set forth in SEQ ID NO: 16 and SEQ ID NO: 22).

IgG1-huCD3-H101G-FEAL (having the VH and VL sequences set forth in SEQ ID NO: 17 and SEQ ID NO: 22).

The annotation IgG1 indicates that full length antibodies of the IgG1 isotype were made, and the FEAL
10 annotation indicates that the heavy chain constant regions contains amino acid substitutions L234F, L235E, D265A and F405L and the light chain constant regions were of the lambda type (SEQ ID NO. 60 and 64, respectively).

Control antibodies

IgG1-b12-K409R (having the VH and VL sequences set forth in SEQ ID NO: 14 and SEQ ID NO: 15).

15 The annotation IgG1 indicates that full length antibodies of the IgG1 isotype were made, and the K409R annotation indicates that the heavy chain constant regions contains amino acid substitution K409R and the light chain constant regions were of the kappa type (SEQ ID NO. 62 and 63, respectively).*Bispecific antibodies*

The CD3 and B7H4 antibodies described above were combined to generate a bispecific antibody,
20 having one antigen-binding region capable of binding human CD3 and the other antigen-binding region capable of binding B7H4, providing a bispecific antibody of the isotype IgG1, which is annotated as bsIgG1.

bsIgG1-huCD3-FEALxB7H4-C1-FEAR

bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR

25 bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR

bsIgG1-huCD3-FEALxB7H4-C2-FEAR

bsIgG1-huCD3-FEALxB7H4-C3-FEAR

bsIgG1-huCD3-FEALxB7H4-C4-FEAR

bsIgG1-huCD3-H101G-FEALxB7H4-C2-FEAR

bsIgG1-huCD3-H101G-FEALxB7H4-C3-FEAR

bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR

bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR

bsIgG1-huCD3-FEALxb12-FEAR (for the b12 arm having the VH and VL sequences set forth in SEQ ID
5 NO: 14 and SEQ ID NO: 15)

bsIgG1-huCD3-H101G-FEALxb12-FEAR

Example 7 - Determining the B7H4 domain and functional epitope involved in binding using B7H4-B7H3 chimeric molecules and a B7H4 alanine scanning library

Domain mapping using B7H4-B7H3 chimeric molecules using end-point analysis

10 The B7H4 domain specificity of the B7H4 antibodies was determined using a panel of cells transfected to express human B7H4, human B7H3 (a structurally comparable protein with sufficient amino acid sequence difference in the extracellular domain) or two different human B7H4-B7H3 chimeric molecules. Expression constructs were prepared encoding human B7H4, human B7H3 (Uniprot accession no. Q5ZPR3-1; SEQ ID NO: 9), or a chimeric molecule containing the IgV domain of B7H3 and
15 the IgC domain of B7H4 (B7H3-IgV/B7H4-IgC; SEQ ID NO: 11), or a chimeric molecule containing the IgV domain of B7H4 and the IgC domain of B7H3 (B7H4-IgV/B7H3-IgC ; SEQ ID NO: 10). HEK cells were transiently transfected to express these constructs.

Cells (3x10^4 cell/well) were incubated in polystyrene 96-well round-bottom plates (Greiner bio-one, cat. no. 650101) with serial dilutions of antibodies (range 0.0046 to 10 µg/mL in 3-fold dilution steps)
20 in 50 µL PBS/0.1% BSA/0.02% azide (FACS buffer) at 4°C for 30 min. After washing twice in FACS buffer, cells were incubated with secondary antibody at 4°C for 30 min. As a secondary antibody, R-Phycoerythrin (PE)-conjugated goat-anti-human IgG F(ab')$_2$ (1:500 in staining buffer; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, cat. no. 109-116-098) was used. Next, cells were washed twice in FACS buffer, re-suspended in 20 µL FACS buffer and analyzed on an iQue Screener
25 (Intellicyt Corporation, USA). The binding of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR, bsIgG1-huCD3-FEALxB7H4-C4-FEAR, bsIgG1-huCD3-FEALxB7H4-C3-FEAR and bsIgG1-huCD3-FEALxB7H4-C2-FEAR at 10 µg/mL was determined as % mean fluorescence intensity (MFI) of the binding 10 µg/mL of:

- IgG1-B7H3-BRCA84D (a B7H3-specific IgG1 antibody, generated as described above with CDR sequences as described for antibody BRCA84D in WO2011109400) to B7H3 expressing cells,
30 • bsIgG1-huCD3-FEALxB7H4-C4-FEAR to B7H3-IgV/B7H4-IgC expressing cells,
- bsIgG1-huCD3-FEALxB7H4-C2-FEAR to B7H4-IgV/B7H3-IgC expressing cells,
- and bsIgG1-huCD3-FEALxB7H4-C3-FEAR to B7H4 expressing cells.

Figure 1 shows that the IgC domain of B7H4 is involved in binding of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-FEALxB7H4-C4-FEAR, both the IgC and IgV domain of B7H4 are involved in binding of bsIgG1-huCD3-FEALxB7H4-C3-FEAR, and at least the IgV domain of B7H4 is involved in binding of bsIgG1-huCD3-FEALxB7H4-C2-FEAR. For the C2 antibody from which the variable domains
5 were used to created bsIgG1-huCD3-FEALxB7H4-C2-FEAR, it has been described that it binds to the IgV domain; the data in Figure 1 indicates that the IgC domain is also involved in binding (WO2014159835 and Leong et al 2015, Mol. Pharmaceutics 12, 1717–1729).

Domain mapping using B7H4-B7H3 chimeric molecules using analysis of full dose-response curves

Further experiments were conducted to study the B7H4 domain specificity of the B7H4 antibodies in
10 more detail, by analysis of full dose-response curves. In these experiments, the domain specificity of bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR was also determined. Binding of serial dilutions (0.014 to 30 µg/mL in 3-fold dilution steps) of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C2-FEAR, bsIgG1-huCD-H101G-FEALxB7H4-C3-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR to HEK cells transiently
15 transfected to express human B7H4 or the B7H4-B7H3 chimeric molecules B7H3-IgV/B7H4-IgC or B7H4-IgV/B7H3-IgC was determined as described above. Figure 2 shows the dose-response curves, showing that the IgC domain of B7H4 is involved in binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, in line with the findings of the alanine scanning library experiments. Furthermore, the IgV domain is involved in the binding of bsIgG1-huCD3-H101G-FEALxB7H4-C2-FEAR, bsIgG1-huCD3-
20 H101G-FEALxB7H4-C4-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR, whereas both the IgC and IgV domain appear involved in the binding of bsIgG1-huCD3-H101GFEALxB7H4-C3-FEAR.

Determination of the contribution of B7H4 amino acid residues to binding of B7H4 antibodies using a B7H4 alanine scanning library

Library design

25 A human B7H4 (Uniprot Q7Z7D3-1) single residue alanine library was synthesized (GeneArt) in which all amino acid residues in the extracellular domain of human B7H4 were individually mutated to alanines except for positions containing alanines or cysteines. Cysteines were not mutated to minimize the chance of structural disruption of the antigen. The library was cloned in the pMAC expression vector containing a CMV/TK-polyA expression cassette, an Amp resistance gene and a pBR322
30 replication origin.

Library production and screening

The antibodies C1-N52S, C2 and C3 were generated as recombinant monovalent antibodies as described in WO2007059782 with a mNeonGreen tag. The wild type B7H4 and alanine mutants were expressed individually in FreeStyle HEK293 cells according to the manufacturer's instructions (Thermo Scientific). One day post transfection the cells were harvested. Approximately 50,000 cells were
5 incubated with 20 µL mNeoGreen labeled antibody of interest. Cells were incubated for 1 hour at room temperature. Subsequently, 150 µL FACS buffer was added and cells were washed twice with FACS buffer. Cells were resuspended in 30 µL fresh FACS buffer and analyzed by flow cytometry using an iQue Screener (Intellicyt Corporation, USA).

The entire experiment was performed 2 times in duplicate.

10 Data analysis

For every sample, the average antibody binding per cell was determined as the geometric mean of the fluorescence intensity (gMFI) for the ungated cell population. The gMFI is influenced by the affinity of the antibody for the B7H4 mutant and the expression level of the B7H4 mutant per cell. Since specific alanine mutations can impact the surface expression level of the mutant B7H4, and to correct for
15 expression differences for each B7H4 mutant in general, data were normalized against the binding intensity of a non-cross blocking B7H4 specific reference antibody, using the following equation:

$$\boldsymbol{Normalized\ gMFI_{aa\ position} = \frac{gMFI_{Test\ Ab}}{gMFI_{Reference\ Ab}}}$$

in which C2 was used as reference antibody for C1-N52S and C3, and C1-N52S was used as reference
20 antibody for C2, and in which 'aa position' refers to either a particular ala mutant of B7H4 or wild type (wt) B7H4.

To express loss or gain of binding of the antibodies on a linear Fold Change scale, the following calculation was used:

$$\boldsymbol{Fold\ Change = Log_{10}\left(\frac{Normalized\ gMFI_{ala\ mutant}}{Normalized\ gMFI_{wt}}\right)}$$

25 Gain of binding in most cases will be caused by loss of binding of the reference antibody to specific ala mutants.

Upon these calculations, amino acid positions for which, upon replacing the amino acid with alanine, there is no loss or gain of binding by a particular antibody will give as result '0', gain of binding will result in '>0' and loss of binding will result in '<0'. To correct for sample variation, only B7H4 amino

acid residues where the Fold Change in binding was lower than the mean Fold Change – 1.5 x SD, where SD is the standard deviation of calculated fold changes from four independent experiments for a particular test antibody, were considered 'loss of binding mutants'.

In case the gMFI of the reference antibody for a particular B7H4 mutant was lower than the mean gMFI - 2.5 x SD of the mean $gMFI_{Control\ Ab}$, data were excluded from analysis (as for those B7H4 mutants it was assumed expression levels were not sufficient).

Figure 3 shows the Fold Change in binding of the B7H4 antibodies to B7H4 variants with ala mutations in the ECD, with the amino acid residues where the Fold Change in binding was lower than the mean Fold Change – 1.5 x SD annotated. The Fold Change is indicated in figure 3 as Z-score. The results indicate that:

- binding of antibody C1-N52S is at least dependent on aa S151, V157, D158, Y159, E164, L166, W173, P175, P177, V179, W181, F199, M208, V210, T222, Y223, V240, E242 and I245, which are in the IgC domain of human B7H4,
- binding of antibody C2 is at least dependent on aa R98, G99, R116, K118, N119 and D124, which are in the IgV of human B7H4, and
- binding of antibody C3 is at least dependent on aa N156, E164, V217 and R248, which are in the IgC domain of human B7H4, and
- antibodies C1-N52S, C2 and C3 recognize distinct functional epitopes on B7H4.

Example 8 - Binding of B7H4 monospecific and CD3xB7H4 bispecific antibodies to B7H4 from various species

First, binding of bispecific CD3xB7H4 antibodies and monospecific B7H4 antibodies to HEK-293F cells transiently transfected with human B7H4 or with cynomolgus monkey (*Macaca fascicularis*) B7H4 was analyzed by flow cytometry. Non-transfected HEK-293F cells were used as negative control; these cells were (also) confirmed not to express CD3.

Cells ($3x10^4$ cells/well) were incubated in polystyrene 96-well round-bottom plates (Greiner bio-one, cat. no. 650180) with serial dilutions of antibodies (ranging from 0.000458 to 30 µg/mL in 4-fold dilution steps) in 100 µL PBS/0.1% BSA/0.02% azide (staining buffer) at 4°C for 30 min. Experiments were performed in technical duplicate. After washing twice in staining buffer, cells were incubated in 50 µL secondary antibody at 4°C for 30 min. As a secondary antibody, R-Phycoerythrin (PE)-conjugated goat-anti-human IgG $F(ab')_2$ (1:500 in FACS buffer; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, cat. no. 109-116-098), was used. Cells were washed twice in staining buffer, re-suspended in 30 µL FACS buffer containing Topro-3 (1:10,000 dilution) and analyzed on an iQue Screener

(Intellicyt Corporation, USA). Binding curves were analyzed using non-linear regression (sigmoidal dose-response with variable slope) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

Figure 4 shows that both IgG1-B7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-
5 FEAR bound to cells expressing human B7H4 or cynomolgus monkey B7H4.

Next, binding to HEK-293F cells transiently transfected with B7H4 from dog, rabbit, rat, mouse or pig was determined as described above. Figure 5 shows that IgG1-B7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR bound to B7H4 from dog, rabbit, rat and mouse to varying degrees; for each the apparent affinity (EC50) of the bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR
10 was lower than that of IgG1-B7H4-C1-N52S-FEAR. bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR was not able to bind to pig B7H4, while IgG1-B7H4-C1-N52S-FEAR bound weakly and only at the highest antibody concentrations tested.

The EC50s for binding to human and cynomolgus monkey B7H4 of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR and IgG1-B7H4-C1-N52S-FEAR were in a similar range.

15 Similar studies were performed to compare binding of IgG1-B7H4-C1-052S-FEAR, IgG1-B7H4-C3-FEAR, IgG1-B7H4-C4-FEAR,IgG1-B7H4-C2-FEAR and IgG1-B7H4-C5-FEAR to B7H4 from different species (human, cynomolgus, mouse, rat, rabbit, dog, and pig). Figure 6 shows that binding to HEK cells transfected with human and cynomolgus B7H4 was similar for the tested antibodies. Similar results were obtained with cells expressing rabbit and dog B7H4. However, binding to mouse B7H4 of IgG1-
20 B7H4-C1-N52S-FEAR appeared lower relative to the binding of IgG1-B7H4-C3-FEAR, IgG1-B7H4-C4-FEAR,IgG1-B7H4-C2-FEAR, and IgG1-B7H4-C5-FEAR, which is in conformity with the results in example 3. Also, binding of gG1-B7H4-C1-N52S-FEAR and IgG1-B7H4-C3-FEAR to rat B7H4 appeared lower relative to IgG1-B7H4-C4-FEAR, IgG1-B7H4-C2-FEAR, and IgG1-B7H4-C5-FEAR. Furthermore, while IgG1-B7H4-C4-FEAR,IgG1-B7H4-C2-FEAR and IgG1-B7H4-C5-FEAR bound to pig B7H4, binding of IgG1-
25 B7H4-C1-052S-FEAR was very weak and only apparent at the highest antibody concentration tested. Binding of IgG1-B7H4-C3-FEAR to pig B7H4 was undetectable.

Example 9 - Binding of B7H4 monospecific and CD3xB7H4 bispecific antibodies to B7H4-expressing human tumor cell lines

Binding of IgG1-B7H4-C1-N52S-FEAR and/or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR and/or
30 bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR to the B7H4-expressing human tumor cell lines MCF-7 (breast adenocarcinoma; ATCC, cat. No. HTB-22), MDA-MB-468 (breast adenocarcinoma; ATCC, cat. no. HTB-132) and SK-BR3 (breast adenocarcinoma; ATCC, cat. No. HTB-30), and of bsIgG1-huCD3-

H101G-FEALxB7H4-C1-N52S-FEAR to B7H4-expressing human tumor cell lines NIH-OVCAR-3 (ovarian adenocarcinoma; ATCC, cat. no. HTB-161) or HCC1954 (breast ductal carcinoma; ATCC, cat. no. CRL-2338) was determined. Furthermore, binding of IgG1-B7H4-C1-N52S-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, IgG1-B7H4-C2-FEAR, bsIgG1-huCD3-FEALxB7H4-C2-FEAR or bsIgG1-huCD3-H101G-FEALxB7H4-C2-FEAR, IgG1-B7H4-C3-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C3-FEAR, IgG1-B7H4-C4-FEAR, bsIgG1-huCD3-H101G-FEALxB7H4-C4-FEAR, IgG1-B7H4-C5-FEAR, and/or bsIgG1-huCD3-H101G-FEALxB7H4-C5-FEAR to MDA-MB-468 and HCC1954 cells was determined. Solid tumor cell lines typically do not express CD3. As negative control, tumor cell line HeLa that showed no detectable B7H4 expression (cervix adenocarcinoma; ATCC, cat. no. CCL-2) was used. Binding was analyzed by flow cytometry as described above.

Figure 7 shows that IgG1-B7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR showed comparable dose-dependent binding to MCF-7 and MDA-MB-468 cells, with comparable maximum binding levels.

Figure 8 shows dose-dependent binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR to NIH-OVCAR-3 and HCC1954 cells, and lack of detectable binding to a non-B7H4 expressing cell line, HeLa.

Binding of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR to B7H4-expressing tumor cells was compared using MDA-MB-486 and SK-BR3 cells. Figure 9 shows that bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR showed comparable dose-dependent binding to these cells, with comparable maximum binding levels.

Figure 10 shows dose-dependent binding of the C1-N52S, C2, C3, C4, and C5 B7H4 antibodies in homodimer or bispecific antibody format to MDA-MB-468 and HCC1954 cells. The antibodies based on C4 and C5 showed most efficient binding, the antibodies based on C1-N52S and C2 showed intermediate binding efficiency, and the antibodies based on C3 showed the lowest binding efficiency. Maximum binding was comparable between the antibodies based on C1-N52S, C2, C4 and C5, but lower for the antibodies based on C3.

Example 10 - Binding of B7H4 antibody to primary tumor cells

Primary tumor cells from an ovarian cancer patient were obtained from Discovery Life Sciences (Huntsville, AL, USA; patient ID 110045042). Binding of IgG1-B7H4-C1-N52S-FEAR to tumor cells was assessed by flow cytometry: cells were seeded at $2x10^4$ cells/well in polystyrene 96-well round-bottom plates (Greiner bio-one, cat. no. 650180), centrifuged and incubated with 50 µl Fixable Viability Stain FVS-BV510 (BD Biosciences, cat. no. 564406), 1:1000 diluted in PBS, at 4°C for 30 min. After washing

in staining buffer, cells were incubated with FITC-labeled IgG1-B7H4-C1-N52S-FEAR and a panel of CD3 (EF450 labeled; eBioscience, cat. no. 48-0037-42), CD45 (BV786 labeled; Biolegend, cat. no. 304048), CD14 (PE-Cy7 labeled; BD Biosciences, cat. no. 557742), CD86 (PerCP-Cy5.5 labeled; Biolegend, cat. no. 305420), CD163 (APC-Cy7 labeled; Biolegend, cat. no. 333622) and EpCAM (AF700 labeled; R&D
5 systems, cat. no. FAB9601N) specific antibodies, at 4°C for 30 min. After washing cells were resuspended in staining buffer and analyzed using a FACS Fortessa (BD Biosciences). Single cells were gated based on scatter FSC/SSC and live cells were identified by exclusion of FVS-BV510 positive cells. Tumor cells were identified as EpCAM positive cells.

Flow cytometric analysis showed that IgG1-B7H4-N52S-FEAR bound EpCAM-positive live tumor cells
10 but not to monocytes or T cells within a dissociated tumor cell suspension of an ovarian cancer sample.

Example 11 - Induction of T cell mediated cytotoxicity *in vitro* by CD3xB7H4 bispecific antibodies, using purified T cells as effector cells at varying effector to target ratios

To determine the efficiency of the T cell-mediated tumor cell kill in presence of bispecific antibodies bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, an *in*
15 *vitro* cytotoxicity assay was performed using B7H4-positive tumor cell lines as target cells and purified T cells as effector cells, with varying effector to target cell (E:T) ratios.

T cells were obtained from healthy human donor buffy coats (Sanquin, Amsterdam, The Netherlands) and isolated using the RosetteSep™ human T cell enrichment cocktail (Stemcell Technologies, France, cat. no. 15061) according to the manufacturer's instructions. SK-BR3 cells (16,000 cells/well) were
20 seeded into flat bottom 96-well plates (Greiner-bio-one, The Netherlands, cat. no. 655180) and left to adhere for 4 hours at 37°C. T cells were added to tumor cells at an effector to target (E:T) ratio of 2:1, 4:1 or 8:1. Serial dilutions of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR were added (final concentration ranging from 10,000 to 0.0128 ng/mL; 5-fold dilutions) and plates were incubated for 72 hours at 37°C. Plates were washed 3 times with PBS,
25 and cells were incubated with 150 µl/well of 10% alamarBlue(r) solution (Invitrogen, cat. no. DAL1100) for 4 hours at 37°C. As a positive control for cytotoxicity, cells were incubated with 16 µg/mL phenylarsine oxide (PAO; Sigma-Aldrich, cat. no. P3075; dissolved in dimethylsulfoxide [DMSO; Sigma-Adrich, cat. no. D2438]). AlamarBlue fluorescence, as a measure of metabolic activity of the tumor cell cultures and thus of viable tumor cells, was measured at 615 nm (OD615) on an EnVision plate reader
30 (PerkinElmer). The absorbance of PAO-treated tumor cell samples was set as 0% viability and the absorbance of untreated tumor cell samples was set as 100% viability. The 'percentage viable cells' was calculated as follows:

% viable cells= ([absorbance sample – absorbance PAO-treated target cells]/ [absorbance untreated target cells – absorbance PAO-treated target cells]) x 100.

Dose-response curves and IC50 values were generated using non-linear regression analysis (sigmoidal 5 dose-response with variable slope) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

Figure 11 shows that T cell mediated cytotoxicity was observed at all E:T ratio's, with maximal tumor cell killing (less than 10% viable tumor cells) observed at an E:T ratio of 8:1.

Example 12 - Induction of cytotoxicity *in vitro* in various tumor cell lines by CD3xB7H4 bispecific 10 antibodies and correlation with B7H4 expression level.

The T cell-mediated kill of bispecific antibodies bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR of various B7H4 expressing tumor cell lines was determined in an *in vitro* cytotoxicity assay as described above, using an E:T ratio of 8:1. The following cell lines were used: MCF-7, MDA-MB-486, SK-BR3, NIH-OVCAR-3,HCC1954, and NCI-H1650. From each 15 incubation, 150 µL supernatants containing T cells was transferred to U-bottom 96 Well culture plates (CellStar, cat. no. 650180) prior to washing and alamarBlue incubation (to determine T cell activation and cytokine release, as described below)

For these tumor cell lines, the expression of B7H4 was quantified by quantitative flow cytometry (Human IgG calibrator, BioCytex) according to the manufacturer's instructions, using bsIgG1-huCD3-20 H101G-FEALxB7H4-C1-N52S-FEAR to detect B7H4.

Figure 12 shows both bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR induced dose-dependent T cell mediated cytotoxicity in MCF-7, MDA-MB-486, SK-BR3, NIH-OVCAR-3 and HCC1954 cells in vitro. While maximum cytotoxic activity (<10 % viable tumor cells) was achieved for both bsAb variants, this occurred at lower concentrations for bsIgG1-25 huCD3-FEALxB7H4-C1-N52S-FEAR in comparison with bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (Table 13).

No significant relation between tumor cell lysis and the level of B7H4 expression (Figure 13A) was observed for either bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (Figure 13B). Figure 13B shows the IC50 of T cell-mediated kill, using T cells derived 30 from 4-6 donors, in the presence of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR for each cell line, with the cell lines arranged from lowest to highest level of B7H4 expression. This means that T cell mediated killing can occur over a wide range of B7H4

expression levels.

Table 13 summarizes results across a panel of 5 cell lines and 4 donors.

Table 13. Induction of cytotoxicity *in vitro* in various tumor cell lines by CD3xB7H4 bispecific antibodies.

IC50 range (4 donors each cell line) (µg/ml)				
	CD3-H101GXB7H4		**CD3xB7H4**	
cell line	**lowest**	**highest**	**lowest**	**highest**
MCF7	0.55	1.29	0.012	0.025
OVCAR3	0.09	1.629	0.003	0.012
NCI-H16650	1.67	5.07	N.D.	N.D.
MDA-MB-468	0.08	0.16	0.001	0.004
HCC1954	0.06	0.22	0.001	0.008
SK-BR3	0.09	0.22	0.002	0.016

5

bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR also induced dose-dependent T-cell mediated cytotoxicity of the tested NCI-H1650 NSCLC cell line.

<u>Example 13 - Induction of T cell activation and cytokine production *in vitro* by CD3xB7H4 bispecific antibodies in the presence of B7H4-positive tumor cells</u>

10 The U-bottom 96 well culture plates containing the supernatants collected during the in vitro T cell-mediated cytotoxicity experiments described in example 12 were centrifuged (300 x g) for 3 min at 4°C, after which 75 µL of supernatant was transferred to a new plate for cytokine production measurement, and T cells were kept to assess T cell activation (described below). Cytokine production was analyzed by a multiplex U-plex assay (MeSo Scale Discovery, USA, cat. no. K15049K) according to 15 manufacturer's instructions.

T cells were stained for T cell markers CD3 (1:200; eBioscience, clone OKT3, conjugated to eFluor450), CD4 (1:50; eBioscience, clone OKT4, conjugated to APC-eFluor780), CD8 (1:100; Biolegend, clone RPA-T8, conjugated to AF700) and T cell activation markers CD69 (1:50; BD Biosciences, clone AB2439, conjugated to APC), CD25 (1:50; eBioscience, clone BC96, conjugated to PE-Cy7) and CD279/PD1 (1:50; 20 Biolegend, clone EH12.2H7, conjugated to BV605). Single stained samples with Ultracomp beads (5 µL; Invitrogen, cat. no. 01-2222-42) were included and used for compensation adjustments of the flow cytometer. After 30 min of incubation at 4°C, plates were washed three times with PBS/0.1% BSA/0.02% azide (staining buffer). Cells were resuspended in 120 µL staining buffer and analyzed using

a FACS Fortessa (BD Biosciences). Data were processed using FlowJo (BD Biosciences).

Dose-response curves, EC50, EC90 and EC99 values were calculated using non-linear regression analysis (sigmoidal dose-response with variable slope) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

5 Figure 14A shows T cell activation in the presence of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR for the B7H4-positive tumor cell lines, as defined by the expression of activation markers CD69 on CD8+ T cells (determined by flow cytometry). Figure 14B shows the EC50 of T cell activation, using T cells derived from 3-4 donors, for each of the tumor cell lines.

10 Overall, a subset (approximately 20-50% at the highest antibody concentration) of CD8+ T cells became activated in the presence of either bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR or bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR. T cell activation induced by bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR generally occurred at higher concentrations than that induced by bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (Figure 14A). The EC50 of T cell activation for both bispecific
15 antibodies was variable between target cell line used and between donors (Figure 14B).

Production of cytokines was assessed in supernatants of the tumor cell-T cell cultures by Mesoscale Discovery U-plex multiplex ELISA. Of the 10 cytokines analyzed across the cell line panel, using T cells from 4 donors, significant increases in cytokine levels were primarily observed for IFN-gamma and IL-8 (>2000 pg/ml). IL-4, IL-6 and IL-13 were modulated at much lower levels (<500 pg/ml), while IL-
20 1beta, IL-2, IL-10, IL-12p70, and TNFalpha levels were generally below 50 pg/ml. Because IFN-gamma changes were robustly and consistently detected and IFN-gamma is one of the core cytokines elevated in serum of patients with cytokine release syndrome, the data for this cytokine is represented.

Figure 15 shows the levels of IFN-gamma in the supernatant of T cell-tumor cell co-cultures at antibody concentrations that induced T cell mediated cytotoxicity in 50%, 90% and 99% of tumor cells (EC50,
25 EC90, EC99, resp) in the presence of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, using T cells from at least 3 donors analyzed per cell line. Cytokine production levels varied per donor and per target tumor cell line. Nevertheless, at antibody concentrations that induced the same level (%) of tumor cell killing, in general lower cytokine production levels were seen after exposure of T cell-tumor cell co-cultures to bsIgG1-huCD3-H101G-
30 FEALxB7H4-C1-N52S-FEAR compared to that after exposure to bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR. Thus, at the same level of tumor cell killing, incubation with bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR resulted in lower cytokine production than bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR.

Example 14 – Non-clinical safety studies of CD3xB7H4 bispecific antibodies in cynomolgus monkeys

The non-clinical safety profile of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR was evaluated in non-human primates (cynomolgus monkeys, Macaca fascicularis, originating from Mauritius) at Citoxlab, France. Cynomolgus monkeys were considered 5 the only relevant species for non-clinical safety studies based on the species-specificity of the CD3 arms of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR and bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, and furthermore due to similar binding of the B7H4 arm to human and cynomolgus B7H4 and further pharmacological findings. These studies were conducted in compliance with animal health regulations (Council Directive No. 2010/63/EU of 22 September 2010 and French decret No. 2013-118 10 of 01 February 2013 on the protection of animals used for scientific purposes).

The aim of the studies were to determine the potential toxicity and toxicokinetics of the CD3xB7H4 bispecific antibodies. Here only the results of the toxicokinetics and the determination of cytokine levels in plasma are described.

In two separate studies, the animals were treated with a single dose of 0.1, 1, 3 or 10 mg/kg bsIgG1-15 huCD3-H101G-FEALxB7H4-C1-N52S-FEAR or bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR (one female animal per dose) by intravenous (IV) infusion. The day of infusion was indicated as Day 1 in the study. Blood samples were obtained twice before dosing and 0.5h, 2h 4h, 12h, 24h and 48h after dosing for evaluation of the toxicokinetic profile and plasma cytokine levels, and additionally 168, 336 and 504 hours after dosing for toxicokinetics.

20 *Cytokine levels*

Plasma samples were analyzed for cytokine levels (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF, IL-12p70, IL-15 and CCL2/MCP1) using Luminex xMAP technology.

BsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR administration to cynomolgus monkey produced only minor changes in plasma cytokine levels, which were considered unrelated to test compound, 25 whereas administration of bsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR resulted in dose-dependent increase of IL-6 and MCP-1 levels, as shown in Figure 16.

The lower cytokine levels produced after treatment with bispecific BsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, as compared with BsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR antibody, may offer an advantage in a clinical setting.

30 *Toxicokinetics*

Plasma concentrations of CD3xB7H4 bispecifics were determined using a generic IgG PK ECLIA method. Toxicokinetic parameters were estimated using Certara Phoenix WinNonlin pharmacokinetic software version 8.1 using a non-compartmental approach consistent with the intravenous infusion injection route of administration. Figure 17 shows that the toxicokinetic profiles of both CD3xB7H4 bispecific antibodies were highly comparable up to 7 days post-dose, with both showing dose-related plasma exposure.

A pharmacokinetic modeling exercise was undertaken to assess whether the projected clinical dose range required by the BsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR variant with lower CD3 affinity would be unsustainably high. A PK model was used that was informed by observations in cynomolgus monkey. The clinical dose range was derived that is expected to give rise to one-week average plasma exposure equal to the EC50 to EC90 for T cell mediated cell kill as observed in vitro. The resulting dose range was considered feasible and this aspect gave no reason *a priori* to favor one type of bispecific antibody over the other (BsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR vs. the BsIgG1-huCD3-FEALxB7H4-C1-N52S-FEAR).

Example 15 – B7H4 expression in various human cancer indications

B7H4 mRNA levels were extracted from the Omicsoft TCGA database and visualized using Oncoland software (Qiagen, USA).

Figure 18 shows the B7H4 mRNA expression levels in a range of primary solid tumors, ranked according to median of the expression. mRNA expression was found in a wide range of cancer indication and varied within each indication, with highest median expression found in uterine carcinosarcoma (UCS), bladder urothelial carcinoma (BLCA), pancreatic adenocarcinoma (PAAD), lung squamous cell carcinoma (LUSC), breast invasive carcinoma (BRCA), uterine corpus endometrial carcinoma (UCEC), ovarian serous cystadenocarcinoma (OV) and cholangiocarcinoma (CHOL).

Protein expression of B7H4 in colon, lung (small cell lung cancer, SCLC and non-small cell lung cancer, NSCLC), stomach, pancreatic, bladder, cervical, head and neck, breast (including triple-negative breast cancer, TNBC), ovarian, esophageal, kidney, prostate and uterine cancer and cholangiocarcinoma, was analyzed by immunohistochemistry (IHC) on tissue microarrays (TMA; all purchased from BioMax). Prior to staining, freshly cut TMA sections (5 µm) were deparaffinized and incubated with Target Retrieval Solution pH9 (DAKO, S2367; 30 min at 97°C, 60 min cool down). B7H4 IHC was performed using a commercial rabbit anti-human B7-H4 monoclonal antibody (clone D1M8I, #14572, Cell Signaling Technologies) at optimal dilution (1:25; final concentration 2.6 µg/mL) for 30 min (RT) on a LabVision autostainer platform. Subsequently, sections were incubated with anti-rabbit IgG polymer

(EnvisionTM FLEX+ rabbit (DAKO, S2022), washed and incubated with DAKO Liquid DAB+ Substrate chromogen system (DAKO, K3468). Hematoxylin (DAKO, S3301) was used to detect nucleated cells. Cytokeratin (to determine the tumor region of interest, ROI) IHC was performed with mouse anti-cytokeratin antibody mix (clones AE1/AE3) on Ventana Benchmark using OptiView detection.
5 Cytokeratin was visualized with DAB and nuclei counterstained with hematoxylin using default Ventana reagents. Stained TMA sections were digitized at 20x magnification on a AxioScan (Zeiss). Initially, manual scoring was performed to determine the average B7H4 staining intensity (negative-low-medium-high) and the percentage of tumor cores with >10% B7H4-positive tumor cells.

Subsequently, automated scoring was performed. The tumor ROI was defined using cytokeratin mask
10 on TMA sections adjacent to those stained for B7H4. B7H4 staining intensity in the tumor ROI was quantified (negative, weak (1), moderate (2) or string (3) and the percentage of B7H4 percentage positive tumor cells (range 0 - 100%) was determined using HALO image analysis software. For each indication, the percentage of tumor cores with >10% B7H4-positive tumor cells was determined.

Table 14 shows B7H4 protein expression determined by IHC analysis of BioMax TMAs. No to very low
15 B7H4 expression was seen in colon, prostate, kidney, and small cell lung cancer samples. In samples from the other indications the B7H4 expression varied, with increasing B7H4 expression found in stomach cancer, pancreatic cancer, cholangiocarcinoma, oesophageal cancer, bladder cancer, non-small cell lung cancer (in particular squamous NSCLC), cervical cancer, head and neck cancer, breast cancer (triple negative breast cancer [TNBC] and non-TNBC), ovarian cancer, and uterine cancer.

20 Table 14. B7H4 protein expression determined by IHC analysis of BioMax TMAs. ND = not determined.

		Manual scoring		**Automated scoring**
Indication (BioMax TMA)		**>10 % B7H4 positive (any intensity, by visual assessment)**	**Staining intensity**	**>10 % B7H4 positive (1+ and above, by digital image analysis)**
Colon cancer (n=64)		0%	Negative	
Lung cancer	SCLC (n=60)	1%	Negative-Low	
NSCLC	AC (n=82)	17%	Low	ND

	SQCC (n=95)	48%	Medium	ND
Stomach cancer (n=90)		17%	Low	
Pancreatic cancer (n=60)		25%	Low	ND
Cholangiocarcinoma (n=98)		31%	Low	16%
Bladder cancer (n=60)		43%	Low-Medium	25%
Cervical cancer (n=60)		52%	Low-Medium	27%
Head and Neck cancer (n=92)		47%	Low-Medium	23%
Breast cancer	all (n=232)	78%	Medium-High	72%
	TNBC (n= 35)	89%	Medium-High	ND
Ovarian cancer (n=74)		82%	Medium-High	68%
Uterine cancer (n=73)		82%	Medium-High	75%
Esophageal cancer (n= 53)		36%	Low	ND
Kidney cancer (n= 83)		9%	Negative	ND
Prostate cancer (n= 57)		1%	Negative	ND

Example 16 – Thermal stability and colloidal stability at various pH values

Various formulations of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (20 mg/mL) were prepared with different pH values using a range of buffering agents (acetate, glutamate, succinate, citrate, 5 histidine, phosphate, and tris), spanning the pH range of 4.0 to 8.0, as illustrated in Table 15.

Table 15. Test formulations with varying pH values

Formulation	Buffer	pH
1	20 mM Acetate	4.0
2		4.5
3	20 mM Glutamate	4.5
4		5.0
5	20 mM Succinate	5.0
6		5.5

7	20 mM Citrate	5.5
8	20 mM Histidine	5.5
9	20 mM Citrate	6.0
10	20 mM Histidine	6.0
11	20 mM Citrate	6.5
12	20 mM Histidine	6.5
13	20 mM Phosphate	6.5
14		7.0
15	20 mM Tris	7.5
16		8.0

Differential scanning fluorimetry (DSF) was performed to determine the effect of each pH and buffer combination on the thermal stability of the antibody. DSF analysis utilizes a thermal ramp from 20-95°C to induce protein unfolding and assesses thermal state transitions by measuring the fluorescence profile of the sample over the course of increasing thermal stress. A conformational state transition can be illuminated when a discrete change in the intrinsic fluorescence profile is observed. This discrete change is primarily due to a change in local chemical environment around hydrophobic aromatic residues (e.g. tryptophan) arising from a conformational shift. Data is reported by comparing the barycentric mean (BCM), which is the mean wavelength of fluorescence emission, at each temperature. As aromatic residues become exposed due to unfolding, the fluorescence emission tend to decrease in energy. This allows for the measurement of a minimum unfolding temperature (T_{onset}), seen at the temperature where BCM increases above noise fluctuations, and melting temperature values (T_m), seen where inflection points arise in the BCM vs temperature curve.

Static light scattering (SLS) measurements are determined at 266 nm and 473 nm to assess protein colloidal stability. The intensity of static light scattering from the lasers used to illuminate the sample is proportional to the presence of particles sized on the same order of magnitude as the incident wavelength. This analysis is therefore sensitive to protein aggregation over the temperature ramp. The static light scattering is measured at 266 nm, to detect smaller aggregate particle sizes, as well as 473 nm, for the detection of larger aggregate species. The onset of aggregation temperature (T_{agg}) is determined from these data, which is the temperature at which the protein begins to aggregate. These data are best analyzed by large changes in count intensity – higher counts indicate more light has been scattered due to the association of protein aggregates. Over an increasing temperature ramp, changes in SLS count is typically attributed to significant protein aggregation, minimal changes in SLS counts are attributed to partial aggregation, and no change in SLS counts over the temperature ramp is indicative of negligible protein aggregation. SLS analysis was performed using Notebook Method NB9828p9, Differential Scanning Fluorimetry, Static Light Scattering, and Capillary- Based Dynamic

Light Scattering.

A trend in both T_{onset} and T_m can be seen relative to pH with higher pH tending to increase thermal stability, as seen in Table 16. At pH 5.0 and higher, most formulations showed T_{onset} ≈ 50°C or above, indicating relatively stable base formulations. By comparing pH and T_{onset} values, it is evident that with
5 higher pH, more thermal energy is required to induce protein unfolding and this trend holds up to ~pH 6.5 where both Tm and Tonset values plateau. It is likely that lower pH shifts surface and solvent accessible amino acid side chain charges leading to induction and disruption of electrostatic interactions that destabilize the native fold of the protein, namely Asp (pK=3.9), Glu (pK=4.2), and His (pK=6.0). SLS T_{agg} values were recorded at two incident wavelengths, 266nm and 473nm respectively,
10 and measured during the temperature ramp from 20-95°C concurrently with DSF. This allowed analysis of propensity for protein aggregation and colloidal stability of the antibody in each formulation. Differences were observed with a tendency of higher pH to possess a higher T_{agg} for both wavelengths; see Table 16.

Table 16. DSF and SLS results

Formulation	Avg. Tonset (°C)	Avg. T_m1 (°C)	Avg. T_m2 (°C)	Aggregation SLS 266nm (°C)	SLS 266nm (Counts)	Aggregation SLS 473nm (°C)	SLS 473nm (Counts)
1	42.4	58.7	83.7	0.0	0.0E+00	0.0	0.0E+00
2	48.1	61.6	N.D.	59.0	1.6E+03	0.0	0.0E+00
3	47.2	59.3	N.D.	58.2	1.5E+03	0.0	0.0E+00
4	50.3	61.3	N.D.	60.2	3.0E+03	59.5	5.0E+02
5	52.0	62.7	N.D.	62.1	5.0E+05	63.1	8.0E+04
6	49.2	61.2	N.D.	59.2	1.0E+05	62.4	1.0E+04
7	49.8	61.5	N.D.	61.6	4.0E+05	62.7	4.0E+04
8	51.9	61.2	N.D.	58.1	2.4E+03	60.1	3.5E+02
9	50.9	63.0	N.D.	61.6	5.0E+05	62.0	9.0E+04
10	53.5	63.0	N.D.	55.3	4.5E+03	57.0	7.0E+02
11	51.9	63.0	N.D.	61.2	5.0E+05	61.7	9.0E+04
12	53.8	63.6	N.D.	59.8	6.0E+03	60.9	8.0E+02
13	53.9	64.1	N.D.	63.4	5.0E+05	62.9	8.0E+04
14	52.4	63.3	N.D.	62.5	5.0E+05	62.1	9.0E+04
15	53.0	63.9	N.D.	64.4	4.5E+05	63.0	6.0E+04
16	53.4	63.4	N.D.	61.5	4.6E+05	63.0	6.0E+04

15

Overall, the DSF data show that pH values lower than 5.0 imply T_{onset} values below 50°C with a diminishing benefit above that value. T_{agg} values obtained from SLS illustrate that formulations

containing acetate, glutamate, succinate, and histidine (also corresponding to the pH range of ≤6.5) had a lower propensity for aggregation and higher thermal onsets of aggregation compared to other buffer systems. Also, higher pH values tend to correlate with deamidation, suggesting a particularly preferred pH range of 5.0 to 6.5.

Example 17 – Thermal stability and colloidal stability with additional excipients

Various formulations of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (20 mg/mL) were prepared with varying pH (glutamate at pH=5.0, succinate at pH=5.5, histidine at pH=5.5, and histidine at pH=6.0) and a set of different excipients (NaCl, arginine, sorbitol, and sucrose), see table 17.

Table 17. Test formulations using different buffers, pH values, and excipients

Formulation	Buffer	pH	Excipient
A	20 mM Glutamate	pH 5.0	150 mM NaCl
B			150 mM Arg
C			250 mM Sorbitol
D			250 mM Sucrose
E	20 mM Succinate	pH 5.5	150 mM NaCl
F			150 mM Arg
G			250 mM Sorbitol
H			250 mM Sucrose
I	20 mM Histidine		150 mM NaCl
J			150 mM Arg
K			250 mM Sorbitol
L			250 mM Sucrose
M		pH 6.0	150 mM NaCl
N			150 mM Arg
O			250 mM Sorbitol
P			250 mM Sucrose

Differential scanning fluorimetry (DSF) and static light scattering (SLS) measurements were performed as described in Example 16 above. As summarized in Table 18 below, formulations with charged excipients (arginine and NaCl; formulations A, B, E, F, I, J, M, N) resulted in lower values for both T_{onset} and T_m as compared to their respective counterparts with non-ionic excipients (sorbitol, sucrose).

Table 18. DSF and SLS results

Formulation	Avg. T_{onset} (°C)	Avg. T_m (°C)	T_{Agg} SLS 266nm (°C)	SLS 266nm (Counts)	T_{Agg} SLS 473nm (°C)	SLS 473nm (Counts)
A	49.7	57.5	60.2	5.0E+05	62.0	1.7E+04
B	48.2	59.8	61.4	4.5E+05	62.0	1.3E+04
C	51.1	62.4	60.5	2.2E+03	N/A	2.1E+01
D	52.4	62.6	61.1	2.1E+03	N/A	2.5E+01
E	52.3	62.9	62.7	5.0E+05	63.6	2.0E+04
F	51.6	61.2	62.9	4.6E+05	63.5	1.6E+04
G	53.2	63.8	65.5	4.6E+05	65.5	1.0E+04
H	54.8	63.9	65.1	4.6E+05	65.8	1.2E+04
I	47.6	60.4	59.7	4.5E+05	61.7	1.0E+04
J	46.7	56.5	60.9	4.1E+05	62.0	8.1E+03
K	52.6	61.9	61.4	2.0E+03	N/A	2.8E+01
L	51.4	62.8	59.7	1.8E+03	N/A	2.0E+01
M	51.7	61.4	61.8	5.0E+05	63.8	2.7E+04
N	48.6	60.7	63.5	4.6E+05	63.6	1.1E+04
O	53.7	64.3	61.4	2.7E+03	N/A	2.0E+01
P	53.0	64.6	62.0	2.5E+03	N/A	2.0E+01

Similarly, as revealed by SLS, non-ionic excipients showed lower degrees of aggregation at 266 nm and no aggregation detected at 473 nm (with the exception of succinate-based formulations). Overall, formulations with non-ionic excipients showed superior thermal stability, with buffering agents ranking histidine, glutamate, and succinate, among the tested formulations.

Example 18 – Solubility, thermal stability, colloidal stability of various formulations

Various formulations of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (20 mg/mL) were prepared according to Table 19 below.

Table 19. Test formulations using different buffers, pH values, and excipients

Formulation	Buffer	pH	Excipient 1	Excipient 2
1	20 mM Glutamate	5.0	Sorbitol, 250 mM	-
2		5.0	Sucrose, 250 mM	-
3		5.5	Sorbitol, 250 mM	-

4	20 mM Histidine	5.5	Sorbitol, 125 mM	NaCl, 75 mM
5		5.5	Arg, 75 mM	NaCl, 75 mM
6		6.0	Sorbitol, 250 mM	-
7		5.5	Sucrose, 250 mM	-
8		6.0	Sucrose, 250 mM	-
9	20 mM Succinate	5.5	Arg, 150 mM	-
10		5.5	NaCl, 150 mM	-
11		5.5	Sorbitol, 250 mM	-
12		5.5	Sucrose, 250 mM	-

The formulations of Table 19 were assessed by Dynamic Light Scattering (DLS) to measure size and size homogeneity.

The data are summarized in Table 20. Average hydrodynamic diameters ranged from 6.4 nm (20 mM glutamate, 250 mM sorbitol, pH 5.0) to 19.6 nm (20 mM histidine, 250 mM sorbitol, pH 6.0). Monomer hydrodynamic diameters ranged from 5.3 nm (20 mM glutamate, 250 mM sorbitol pH 5.0) to 13.1 (20 mM succinate, 150 mM NaCl pH 5.5). Average polydispersity index (PDI) values ranged from 0.06 (20 mM histidine, 125 sorbitol, 75 mM NaCl pH 5.5) to 2.79 (20 mM histidine, 250 mM sorbitol pH 6.0). Monomer %PD ranged from 19.6% to 31.4% indicating relatively low polydispersity.

Table 20. DLS results

Formulation	Overall Average Diameter (nm)	Average PDI	Average Monomer Diameter (nm)	Average Monomer % Polydispersity	Average Monomer % Mass	Modality
1	6.4	0.56	5.3	26.5	100.00	Multimodal
2	8.2	1.05	7.2	27.0	99.99	Multimodal
3	8.9	0.14	6.9	22.1	99.99	Multimodal
4	13.2	0.06	11.8	25.4	100.00	Multimodal
5	12.3	0.11	12.1	19.6	100.00	Multimodal
6	19.6	2.79	6.4	28.8	99.99	Multimodal
7	10.2	0.87	8.0	20.7	99.99	Multimodal
8	10.6	1.43	7.4	25.0	99.97	Multimodal
9	12.8	0.15	11.8	31.4	100.00	Monomodal
10	13.4	0.11	13.1	26.8	100.00	Monomodal
11	11.6	0.07	10.4	23.6	100.00	Monomodal
12	15.8	0.14	12.1	28.3	100.0	Multimodal

Four of the highest five average PDI values were also seen in the four formulations having the lowest and highest pH, respectively (pH 5.0 and pH 6.0), indicating that the pH value plays a significant role in the solubility of the antibody.

Differential scanning fluorimetry (DSF) and static light scattering (SLS) measurements were performed
5 as described in Example 16 above. The results are summarized in Table 21.

Table 21. DSF and SLS results

Formulation	Avg. T_{onset} (°C)	Avg. T_m (°C)	T_{Agg} SLS 266nm (°C)	SLS 266nm (Counts)	T_{Agg} SLS 473nm (°C)	SLS 473nm (Counts)
1	3.0E+03	N/A	54.3	62.3	61.9	N/A
2	1.8E+03	N/A	51.0	62.8	61.3	N/A
3	1.4E+03	N/A	52.3	61.9	61.2	N/A
4	3.0E+05	5.5E+03	49.6	58.4	60.5	63.1
5	3.5E+05	5.5E+03	49.6	56.7	60.2	61.7
6	3.3E+03	N/A	55.6	63.8	63.2	N/A
7	2.0E+03	N/A	54.4	62.7	60.7	N/A
8	1.9E+03	N/A	58.4	64.3	62.8	N/A
9	4.0E+05	6.8E+03	53.0	61.8	62.1	63.7
10	4.5E+05	1.2E+04	53.9	62.4	62.1	63.2
11	4.5E+05	5.5E+03	56.4	63.7	63.9	64.5
12	4.5E+05	6.0E+03	56.3	64.7	63.7	65.7

When comparing T_{onset} and T_m values, the non-charged excipients sucrose and sorbitol tend to improve stability. As for SLS, it is apparent that all formulations containing non-charged excipients and either
10 glutamate or histidine show a lack of aggregation at 473 nm and very low comparative magnitude of aggregation at 266 nm as compared to succinate-based and charged excipient containing formulations.

In addition to the results from DSF measurements, these data further support the role of sucrose and sorbitol as thermally stabilizing excipients. Formulations containing non-ionic excipients and either
15 glutamate or histidine showed an overall better characterization profile throughout the course of the solubility study. In addition, histidine and glutamate buffered formulations with sorbitol or sucrose as excipients showed the fastest exchange and concentration rates during the sample preparation phase, suggesting that nonionic excipients play a role in solubilizing the antibody.

Example 19 – Storage stability of various pharmaceutical compositions

A total of 36 formulations of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR (20 mg/mL) were prepared and subjected to two hold conditions, 5±3 °C and 40±2 °C/75±5% RH, respectively, over a 4-week period. The formulations prepared in this experiment are shown in Table 22.

Table 22. Test formulations using different buffers, pH values, excipients and polysorbate 80

Formulation	Buffer	pH	Excipient 1	Excipient 2	PS80 %
1	20 mM Glutamate	5.0	250 mM Sorbitol	-	0.02
2			125 mM Sorbitol	75 mM NaCl	0.02
3			125 mM Sorbitol	75 mM Arg	0.02
4			150 mM Arg	-	0.02
5			250 mM Sucrose	-	0.02
6		5.2	250 mM Sorbitol	-	0.02
7			125 mM Sorbitol	75 mM NaCl	0.02
8			125 mM Sorbitol	75 mM Arg	0.02
9			150 mM Arg	-	0.02
10			250 mM Sucrose	-	0.02
11	20 mM Succinate		250 mM Sorbitol	-	0.02
12			125 mM Sorbitol	75 mM NaCl	0.02
13			125 mM Sorbitol	75 mM Arg	0.02
14			150 mM Arg	-	0.02
15			250 mM Sucrose	-	0.02
16		5.5	250 mM Sorbitol	-	0.02
17			125 mM Sorbitol	75 mM NaCl	0.02
18			125 mM Sorbitol	75 mM Arg	0.02
19			150 mM Arg	-	0.02
20			250 mM Sucrose	-	0.02
21	20 mM Histidine		250 mM Sorbitol	-	0.02
22			125 mM Sorbitol	75 mM NaCl	0.02
23			125 mM Sorbitol	75 mM Arg	0.02
24			150 mM Arg	-	0.02
25			250 mM Sucrose	-	0.02
26		5.8	250 mM Sorbitol	-	0.02
27			125 mM Sorbitol	75 mM NaCl	0.02
28			125 mM Sorbitol	75 mM Arg	0.02
29			150 mM Arg	-	0.02
30			250 mM Sucrose	-	0.02
31			250 mM Sorbitol	-	0.02
32			125 mM Sorbitol	75 mM NaCl	0.02

33		6.0	125 mM Sorbitol	75 mM Arg	0.02
34			150 mM Arg	-	0.02
35			250 mM Sucrose	-	0.02
36	20 mM Glutamate	5.0	250 mM Sorbitol	-	0.02

Purity of the 4-week timepoint samples for both conditions (5±3°C and 40±2°C/75±5% RH) was determined by size exclusion chromatography (SEC) and these data are summarized in Table 23.

Table 23. Impurities after 4-week storage under different conditions

Formulation	Condition	% Monomer	% HMW	% LMW	% Total Impurities
1	5±3°C	99.08	0.90	0.02	0.92
	40±2°C/75±5% RH	98.51	0.91	0.58	1.49
2	5±3°C	99.02	0.96	0.02	0.98
	40±2°C/75±5% RH	97.32	1.80	0.88	2.68
3	5±3°C	99.02	0.96	0.02	0.98
	40±2°C/75±5% RH	97.70	1.52	0.78	2.30
4	5±3°C	98.94	1.03	0.03	1.06
	40±2°C/75±5% RH	96.28	2.79	0.93	3.72
5	5±3°C	99.04	0.94	0.02	0.96
	40±2°C/75±5% RH	98.38	1.04	0.58	1.62
6	5±3°C	99.11	0.87	0.02	0.89
	40±2°C/75±5% RH	98.49	0.97	0.54	1.51
7	5±3°C	98.96	1.02	0.02	1.04
	40±2°C/75±5% RH	97.67	1.61	0.72	2.33
8	5±3°C	99.01	0.97	0.02	0.99
	40±2°C/75±5% RH	97.94	1.41	0.65	2.06
9	5±3°C	98.91	1.06	0.03	1.09
	40±2°C/75±5% RH	96.26	2.92	0.82	3.74
10	5±3°C	99.04	0.94	0.02	0.96
	40±2°C/75±5% RH	98.29	1.17	0.54	1.71
11	5±3°C	98.92	1.05	0.03	1.08
	40±2°C/75±5% RH	97.58	1.80	0.62	2.42
12	5±3°C	98.84	1.13	0.02	1.16
	40±2°C/75±5% RH	96.97	2.21	0.81	3.03
13	5±3°C	99.03	0.95	0.02	0.97
	40±2°C/75±5% RH	97.67	1.67	0.66	2.33
14	5±3°C	98.95	1.03	0.02	1.05
	40±2°C/75±5% RH	96.47	2.77	0.76	3.53
15	5±3°C	98.92	1.05	0.03	1.08
	40±2°C/75±5% RH	97.46	1.92	0.62	2.54

Formulation	Condition	% Monomer	% HMW	% LMW	% Total Impurities
16	5±3°C	98.76	1.21	0.04	1.24
	40±2°C/75±5% RH	97.89	1.47	0.64	2.11
17	5±3°C	98.65	1.32	0.03	1.35
	40±2°C/75±5% RH	96.83	2.37	0.80	3.17
18	5±3°C	99.00	0.98	0.03	1.00
	40±2°C/75±5% RH	97.92	1.52	0.57	2.08
19	5±3°C	98.95	1.03	0.02	1.05
	40±2°C/75±5% RH	96.81	2.55	0.64	3.19
20	5±3°C	98.81	1.17	0.03	1.19
	40±2°C/75±5% RH	97.54	1.87	0.60	2.46
21	5±3°C	98.75	1.20	0.04	1.25
	40±2°C/75±5% RH	95.53	3.65	0.82	4.47
22	5±3°C	99.06	0.92	0.02	0.94
	40±2°C/75±5% RH	97.68	1.70	0.62	2.32
23	5±3°C	99.00	0.99	0.01	1.00
	40±2°C/75±5% RH	96.43	2.95	0.62	3.57
24	5±3°C	98.93	1.06	0.02	1.07
	40±2°C/75±5% RH	94.85	4.35	0.80	5.15
25	5±3°C	99.07	0.92	0.02	0.93
	40±2°C/75±5% RH	98.24	1.27	0.49	1.76
26	5±3°C	99.07	0.91	0.02	0.93
	40±2°C/75±5% RH	98.39	1.18	0.43	1.61
27	5±3°C	98.95	1.03	0.02	1.05
	40±2°C/75±5% RH	96.78	2.68	0.54	3.22
28	5±3°C	98.89	1.09	0.02	1.11
	40±2°C/75±5% RH	95.53	3.89	0.58	4.47
29	5±3°C	98.85	1.13	0.02	1.15
	40±2°C/75±5% RH	95.26	4.05	0.69	4.74
30	5±3°C	99.03	0.95	0.02	0.97
	40±2°C/75±5% RH	98.07	1.48	0.45	1.93
31	5±3°C	99.09	0.89	0.02	0.91
	40±2°C/75±5% RH	98.34	1.22	0.44	1.66
32	5±3°C	98.92	1.06	0.02	1.08
	40±2°C/75±5% RH	97.11	2.40	0.49	2.89
33	5±3°C	98.87	1.10	0.03	1.13
	40±2°C/75±5% RH	96.23	3.20	0.57	3.77
34	5±3°C	98.73	1.24	0.03	1.27
	40±2°C/75±5% RH	94.28	5.06	0.66	5.72
35	5±3°C	98.98	0.99	0.03	1.02
	40±2°C/75±5% RH	97.76	1.78	0.46	2.24
	5±3°C	99.17	0.80	0.04	0.83

Formulation	Condition	% Monomer	% HMW	% LMW	% Total Impurities
36	40±2°C/75±5% RH	98.56	0.86	0.58	1.44

At the 5±3°C condition, the purity displayed a narrow range from 98.5% to 99.2%. A wider range of 94.3% to 98.6% purity was observed for the 40±2°C/75±5% RH condition. Formulations with 250 mM sorbitol or sucrose as the excipient showed the lowest difference between the two conditions excepting formulation 21 (20 mM histidine, 250 mM sorbitol, pH 5.5).

Among these formulations, a trend is observed with higher purity correlating with lower pH within excipient groups. Sorbitol-based formulations slightly outperformed sucrose formulations as well and both glutamate- and histidine-based formulations fared better than succinate-based counterparts.

Example 20 – Storage stability of two preferred pharmaceutical compositions

The purpose of this example is to investigate the 3, 6 and 12 months stability for bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR at 20 mg/mL in two separate formulations: 20mM glutamate, 250 mM sorbitol, 0.02% PS80, pH 5.2 and 20 mM Histidine, 250 mM sucrose, 0.02% PS80, pH 5.5. These formulations are Formulation 6 and 25, respectively, of Table 22 above.

For preparation of the samples of the two formulations a volume of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR antibody was dialyzed into formulation buffer (20mM glutamate, 250 mM sorbitol, 0.02% PS80, pH 5.2 or 20 mM Histidine, 250 mM sucrose, 0.02% PS80, pH5.5) at room temperature at a minimum buffer to sample volume of 40. Following dialysis, the pH was confirmed to be 5.2 ± 0.1 for formulation 6 and to be 5.5 for formulation 25. The concentration of the material was adjusted to 20 ± 2 mg/mL and supplemented with PS80 to a final concentration of 0.02% and finally filtered through a 0.22 µm PVDF filter in a laminar flow hood. The samples were separately filled into 2-mL Type I glass vials to a volume of 1-mL and placed on stability for up to 12 months.

Vials containing the formulations were stored upright for 12 months at -75°C and for 6 months at 5°C, 25°C/60%RH and 40°C/75%RH. The initial time point (T0) included separate samples conditioned with five freeze/thaw cycles, 48-hour agitation (including control) or typical storage conditions (5°C). Stability samples were analyzed and the results are summarized in Tables 24.

Table 24. Stability after 3, 6 and 12 months storage under different conditions.

Formulation 6: 20 mM Glutamate, 250 mM Sorbitol, 0.02% PS-80, pH 5.2				
Method	**T0**			
	Release	5X Freeze/Thaw	48 Hour Agitation	48 Hour non-agitation (Control)
Appearance (visual)				
Color	Colorless	Colorless	Colorless	Colorless
Clarity	Clear	Clear	Clear	Clear
Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates
pH	5,3	5,2	5,2	5,2
Osmolality (mOsm/kg)	305	302	303	303
Concentration (UV_{280})	19,5	19,5	19,3	19,3
Turbidity (A_{550})	0,00	0,00	0,00	0,00
Particle Count - MFI				
2um	4820	1022	1454	1234
5um	945	123	192	235
10um	247	28	31	55
25um	33	2	4	11
Purity - SEC				
Monomer %	98,3	98,9	99,0	98,9
% HMW	1,0	1,0	0,9	1,0
% LMW	0,1	0,1	0,0	0,1
Total Impurities	1,0	1,1	1,0	1,1
Purity - CE-SDS Non-Reduced				
Purity (%)	96,6	95,5	93,9	94,2
Frontside Impurities (%)	3,1	3,6	4,6	4,8
Backside Impurities (%)	0,3	0,9	1,5	1,0
Total Impurities (%)	3,4	4,5	6,1	5,8
Purity - CE-SDS Reduced				
Purity (%)	99,8	99,7	99,7	99,7
Impurities (%)	0,2	0,3	0,3	0,3
Charge Heterogeneity - icIEF				
Main Peak (%)	41,6	42,9	41,8	42,4
TBV (%)	3,9	2,4	3,1	3,0
TAV (%)	54,5	54,7	55,1	54,7
Cation Exchange Chromatography - CEX				
Main Peak (%)	82,9			

TBV (%)	2,0	
TAV (%)	15,0	

Formulation 6: 20 mM Glutamate, 250 mM Sorbitol, 0.02% PS-80, pH 5.2				
Method	**3 months**			
	-75 ± 10°C	5 ± 3°C	25 ± 2°C	40 ± 2°C
Appearance				
Color	Colorless Liquid	Colorless Liquid	Colorless Liquid	Colorless Liquid
Clarity	Clear Liquid	Clear Liquid	Clear Liquid	Clear Liquid
Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates
pH	5,3	5,2	5,2	5,2
Osmolality (mOsm/kg)	-	--	--	--
Concentration (UV_{280})	20,3	20,6	21,5	19,8
Turbidity (A_{550})	0,00	0,00	0,01	0,01
Particle Count - MFI				
2um	1712	1529	2318	1791
5um	490	356	399	491
10um	160	104	84	168
25um	5	9	19	15
Purity - SEC				
Monomer %	99,0	98,9	98,7	95,8
% HMW	0,9	0,9	0,9	1,9
% LMW	0,1	0,1	0,4	2,3
Total Impurities	1,0	1,1	1,3	4,2
Purity - CE-SDS Non-Reduced				
Purity (%)	95,9	95,7	94,9	88,5
Frontside Impurities (%)	3,9	3,9	3,3	11,3
Backside Impurities (%)	0,1	0,3	0,2	0,2
Total Impurities (%)	4,1	4,3	3,4	11,5
Purity - CE-SDS Reduced				
Purity (%)	99,3	99,3	99,0	92,2
Impurities (%)	0,7	0,7	1,0	7,8
Charge Heterogeneity - icIEF				

Main Peak (%)	43,7	43,4	36,3	9,8
TBV (%)	3,3	3,3	3,7	2,4
TAV (%)	53,0	53,3	60,0	87,8
Cation Exchange Chromatography – CEX				
Main Peak (%)	83,1	84,7	78,0	39,0
TBV (%)	2,7	2,4	2,5	1,5
TAV (%)	14,2	12,8	19,6	59,4

Formulation 6: 20 mM Glutamate, 250 mM Sorbitol, 0.02% PS-80, pH 5.2				
Method	**6 months**			**12 months**
	5 ± 3°C	25 ± 2°C	40 ± 2°C	-75 ± 10°C
Appearance				
Color	Colorless Liquid	Colorless Liquid	Very Slightly Yellow Liquid	Colorless Liquid
Clarity	Clear Liquid	Clear Liquid	Clear Liquid	Clear Liquid
Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates
pH	5,3	5,3	5,3	5,3
Osmolality (mOsm/kg)	-	-	-	--
Concentration (UV_{280})	18,9	19,1	19,1	20,0
Turbidity (A_{550})	0,02	-0,02	0,02	0,01
Particle Count - MFI				
2um	2696	963	3265	1602
5um	304	78	444	233
10um	36	4	40	40
25um	6	4	4	6
Purity - SEC				
Monomer %	98,9	98,1	84,3	99,0
% HMW	0,9	1,2	4,7	0,8
% LMW	0,1	0,7	11,0	0,1
Total Impurities	1,1	1,9	15,7	1,0
Purity - CE-SDS Non-Reduced				
Purity (%)	94,7	92,2	76,0	97,6
Frontside Impurities (%)	4,9	7,4	23,4	1,9

Backside Impurities (%)	0,4	0,4	0,7	0,5
Total Impurities (%)	5,3	7,8	24,0	2,4
Purity - CE-SDS Reduced				
Purity (%)	99,2	97,7	81,8	99,5
Impurities (%)	0,8	2,3	18,2	0,5
Charge Heterogeneity - icIEF				
Main Peak (%)	39,3	27,2	4,6	43,3
TBV (%)	3,8	4,1	Not Detected	3,2
TAV (%)	56,9	68,6	95,4	53,6
Cation Exchange Chromatography – CEX				
Main Peak (%)	81,9	66,2	12,4	82,2
TBV (%)	2,2	2,6	1,4	2,2
TAV (%)	15,9	31,1	86,3	15,6

Formulation 25: 20 mM Histidine, 250 mM Sucrose, 0.02% PS-80, pH 5.5				
Method	**T0**			
	Release	5X Freeze/Thaw	48 Hour Agitation	48 Hour non-agitation (Control)
Appearance				
Color	Colorless	Colorless	Colorless	Colorless
Clarity	Clear	Clear	Clear	Clear
Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates
pH	5,5	5,4	5,5	5,4
Osmolality (mOsm/kg)	299	296	295	297
Concentration (UV_{280})	19,2	19,3	19,1	19,4
Turbidity (A_{550})	0,01	0,04	0,02	0,01
Particle Count - MFI				
2um	1061	3257	1510	775
5um	146	593	237	96
10um	32	151	64	24
25um	3	28	16	4
Purity - SEC				
Monomer %	99,0	99,0	97,8	98,9
% HMW	1,0	1,0	0,9	0,9

% LMW	0,1	0,1	0,1	0,1
Total Impurities	1,1	1,1	1,0	1,1
Purity - CE-SDS Non-Reduced				
Purity (%)	96,6	95,5	87,8	95,5
Frontside Impurities (%)	2,8	3,7	10,2	3,9
Backside Impurities (%)	0,5	0,8	2,0	0,6
Total Impurities (%)	3,4	4,5	12,2	4,5
Purity - CE-SDS Reduced				
Purity (%)	99,7	99,7	99,7	99,7
Impurities (%)	0,3	0,3	0,3	0,3
Charge Heterogeneity - icIEF				
Main Peak (%)	42,0	42,2	42,4	42,4
TBV (%)	3,2	3,0	3,0	3,1
TAV (%)	54,8	54,8	54,6	54,6
Cation Exchange Chromatography - CEX				
Main Peak (%)	82,8			
TBV (%)	2,0			
TAV (%)	15,2			

Formulation 25: 20 mM Histidine, 250 mM Sucrose, 0.02% PS-80, pH 5.5				
Method	**3 months**			
	-75 ± 10°C	5 ± 3°C	25 ± 2°C	40 ± 2°C
Appearance				
Color	Colorless Liquid	Colorless Liquid	Colorless Liquid	Slightly Yellow
Clarity	Clear Liquid	Clear Liquid	Clear Liquid	Clear Liquid
Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates
pH	5,4	5,4	5,4	5,4
Osmolality (mOsm/kg)	--	--	--	--
Concentration (UV_{280})	19,6	19,9	19,6	19,7
Turbidity (A_{550})	0,00	-0,02	-0,02	-0,02
Particle Count - MFI				
2um	1303	2176	2607	3732

5um	272	552	766	748
10um	67	196	282	252
25um	3	34	62	61
Purity - SEC				
Monomer %	99,0	99,0	98,8	95,9
% HMW	0,9	0,9	0,9	2,0
% LMW	0,1	0,1	0,3	2,1
Total Impurities	1,0	1,0	1,2	4,1
Purity - CE-SDS Non-Reduced				
Purity (%)	96,0	95,9	95,3	89,8
Frontside Impurities (%)	3,7	3,9	4,6	10,1
Backside Impurities (%)	0,2	0,2	0,2	0,2
Total Impurities (%)	3,9	4,1	4,7	10,2
Purity - CE-SDS Reduced				
Purity (%)	99,0	99,3	99,1	93,3
Impurities (%)	1,0	0,7	0,9	6,7
Charge Heterogeneity - icIEF				
Main Peak (%)	43,0	43,4	37,7	9,4
TBV (%)	3,5	3,2	3,4	1,4
TAV (%)	53,5	53,4	58,9	89,2
Cation Exchange Chromatography – CEX				
Main Peak (%)	85,0	85,9	78,5	56,0
TBV (%)	2,1	1,7	0,7	1,7
TAV (%)	12,9	12,4	20,9	42,2

Formulation 25: 20 mM Histidine, 250 mM Sucrose, 0.02% PS-80, pH 5.5				
Method	**6 months**			**12 months**
	5 ± 3°C	25 ± 2°C	40 ± 2°C	-75 ± 10°C
Appearance				
Color	Colorless Liquid	Very Slightly Yellow Liquid	Slightly Yellow to Yellow	Colorless Liquid
Clarity	Clear Liquid	Clear Liquid	Clear Liquid	Clear Liquid
Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates	Free of Visible Particulates
pH	5,5	5,5	5.4	5,4

Osmolality (mOsm/kg)	-	-	-	--
Concentration (UV_{280})	18,7	18,9	17.6	19,2
Turbidity (A_{550})	-0,30	0,00	-0.01	0,03
Particle Count - MFI				
2um	1859	2195	3529	1715
5um	275	340	403	178
10um	19	34	29	50
25um	2	6	6	10
Purity - SEC				
Monomer %	98,9	98,3	83,1	99,0
% HMW	1,0	1,1	6,4	0,9
% LMW	0,1	0,6	10,6	0,1
Total Impurities	1,1	1,7	16,9	1,0
Purity - CE-SDS Non-Reduced				
Purity (%)	94,9	93,0	77,0	97,9
Frontside Impurities (%)	4,7	6,7	22,4	1,7
Backside Impurities (%)	0,4	0,3	0,7	0,4
Total Impurities (%)	5,1	7,0	23,0	2,1
Purity - CE-SDS Reduced				
Purity (%)	99,0	98,1	83,8	99,6
Impurities (%)	1,0	1,9	16,2	0,4
Charge Heterogeneity - icIEF				
Main Peak (%)	41,5	28,5	3,6	40,3
TBV (%)	3,2	3,9	Not Detected	3,4
TAV (%)	55,3	67,6	96,4	56,3
Cation Exchange Chromatography – CEX				
Main Peak (%)	81,5	68,4	30,0	82,3
TBV (%)	2,2	2,8	2.9	2,1
TAV (%)	16,3	28,9	67.1	15,6

Overall, both formulations demonstrated minimal changes in in the 5X freeze/thaw and 48-hour agitation conditions when compared to the initial (release) sample or respective control. Additionally,

both formulations demonstrated minimal changes in product stability when stored at -75°C through 12 months, 5°C through 6 months and to a slightly lesser extent, 25°C/60%RH for 6 months.

The SEC data shows that formulation 6 is slightly better than formulation 25 at 40°C where the total impurities is at 15.7% for formulation 6 and 16.9% for formulation 25.

5 Overall, the results demonstrated that 20 mg/mL of bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR is stable in 20mM glutamate, 250 mM sorbitol, 0.02% PS80, pH 5.2 or 20 mM Histidine, 250 mM sucrose, 0.02% PS80, pH 5.5 under nominal conditions for up to 12 months.

10

Claims

1. A pharmaceutical composition comprising:

a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3,

5 b) a buffering agent,

wherein the pH of the composition is from 4.0 to 8.0.

2. A pharmaceutical composition according to claim 1 wherein said antigen-binding regions capable of binding to human B7H4 and human CD3 comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions are humanized and/or human.

10 3. A pharmaceutical composition according to claim 1 or 2, wherein the pH of the composition is from 4.5 to 6.5, such as from 5.0 to 6.0, such as from 5.2 to 5.5.

4. A pharmaceutical composition according to any one of the preceding claims, wherein the buffering agent is selected from the group consisting of histidine, glutamate, and mixtures thereof.

5. A pharmaceutical composition according to any one of the preceding claims, wherein the 15 pharmaceutical composition further comprises c) a non-ionic excipient, such as s a sugar or a sugar alcohol.

6. A pharmaceutical composition according to claim 5 , wherein the non-ionic excipient is selected from sorbitol, sucrose or mixtures thereof.

7. A pharmaceutical composition according to any of claims 5-6, wherein the non-ionic excipient is 20 present at a concentration of 100 to 300 mM, such as 125-250 mM preferably 250 mM.

8. A pharmaceutical composition according to any one of the preceding claims wherein the pharmaceutical composition further comprises d) a surfactant.

9. A pharmaceutical composition according to claim 8, wherein the surfactant is selected from the group consisting of glycerol monooleate, benzethonium chloride, sodium docusate, phospholipids, 25 polyethylene alkyl ethers, sodium lauryl sulfate and tricaprylin, benzalkonium chloride, citrimide, cetylpyridinium chloride and phospholipids, alpha tocopherol, glycerol monooleate, myristyl alcohol, phospholipids, poloxamers, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbintan fatty acid esters, polyoxyethylene sterarates, polyoxyl hydroxystearate, polyoxylglycerides, polysorbates, propylene glycol dilaurate, propylene glycol monolaurate, sorbitan 30 esters sucrose palmitate, sucrose stearate, tricaprylin and TPGS, and mixtures thereof.

10. A pharmaceutical composition according to claims 8 or 9, wherein the surfactant is a polysorbate, optionally wherein the polysorbate is polysorbate 20 or 80, preferably polysorbate 80.

11. A pharmaceutical composition according to any of claims 8-10, wherein the surfactant is present at a concentration from about 0.005% to 0.4% w/v, such as from about 0.01 to 0.1 % w/v, such as from about 0.01 to 0.09 % w/v such as from about 0.01 to 0.06 % w/v such as from about 0.01 to 0.05% w/v such as 0.02% w/v or 0.03% w/v or 0.04% w/v or 0.05% w/v, preferably 0.02% w/v.

12. A pharmaceutical composition according to any one of the preceding claims, wherein the concentration of the antibody is 0.5 to 100 mg/ml, such as 1.0 to 50 mg/ml, or such as 5 to 30 mg/ml, such as 5 mg/ml, or 6 mg/ml, or 7 mg/ml, or 8 mg/ml, or 9 mg/ml, or 10 mg/ml, or 11 mg/ml, or 12 mg/ml, or 13 mg/ml, or 14 mg/ml, or 15 mg/ml, or 16 mg/ml, or 17 mg/ml, or 18 mg/ml, or 19 mg/ml, or 20 mg/ml, or 21 mg/ml, or 22 mg/ml, or 23 mg/ml, or 24 mg/ml, or 25 mg/ml, or 26 mg/ml, or 27 mg/ml, or 28 mg/ml, or 29 mg/ml, 30 mg/ml, 31 mg/ml, 32 mg/ml, 33 mg/ml, 34 mg/ml, 35 mg/ml, 36 mg/ml, 37 mg/ml, 38 mg/ml, 39 mg/ml, 40 mg/ml, 41 mg/ml, 42 mg/ml, 43 mg/ml, 44 mg/ml, 45 mg/ml, 46 mg/ml, 47 mg/ml, 48 mg/ml, 49 mg/ml, 50 mg/ml, 51 mg/ml, 52 mg/ml, 53 mg/ml, 54 mg/ml, 55 mg/ml, 56 mg/ml, 57 mg/ml, 58 mg/ml, 59 mg/ml, or such as 60 mg/ml.

13. A pharmaceutical composition according to any one of the preceding claims, wherein the buffering agent is present at a concentration of 5 to 40 mM, such as 10-30 mM, preferably 20 mM.

14. A pharmaceutical composition according to any one of the preceding claims, wherein the pharmaceutical composition is an aqueous composition.

15. A pharmaceutical composition according to any one of the preceding claims, comprising:

a) 5-50 mg/ml of the antibody,

b) 10 to 20 mM glutamate or histidine,

c) 150 to 350 mM sorbitol or sucrose,

d) a polysorbate,

wherein the pH of the composition is from 5.0 to 6.0.

16. A pharmaceutical composition according to any one of the preceding claims, selected from the group consisting of

a pharmaceutical composition comprising a) 10-20 mg/ml of the antibody, b) 20 mM glutamate, c) 250 mM sorbitol, d) 0.02% w/v polysorbate 80, wherein the pH of the composition is from 5.1-5.3, and

a pharmaceutical composition comprising a) 10-20 mg/ml of the antibody, b) 20 mM histidine, c)

250 mM sucrose, d) 0.02% w/v polysorbate 80, wherein the pH of the composition is from 5.4-5.6.

17. A pharmaceutical composition according to any one of the preceding claims, wherein the composition is a liquid composition.

5 18. A pharmaceutical composition according to any claims 1-17, wherein the composition is an intravenous composition, and/or wherein the composition is for use in intravenous administration.

19. A pharmaceutical composition according to any one of the preceding claims, wherein the composition is stable for pharmaceutical use for at least 6 months, such as at least 9 month or at least 12 months at a storage temperature of 2-8°C, such as 5°C.

10 20. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody is a bispecific antibody.

21. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody is a bivalent antibody.

22. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-
15 binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO. 17,

and,

a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID
20 NO: 22.

23. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences of SEQ
25 ID NOs.: 18, 19 and 21

and,

a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences of SEQ ID NO: 23, GTN and 24, respectively.

30 24. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 17, or a sequence

having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 17;

and,

a light chain variable region (VL) comprising the sequence of SEQ ID NO: 22 or a sequence having at least 90%, at least 95%, at least 97%, or at least 99% amino acid sequence identity to the sequence of SEQ ID NO: 22.

25. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region capable of binding to human B7H4 comprises:

a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 25 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 36 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 40;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 43 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 47;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 50 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO.54; or

f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 31 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 65 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 69.

26. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region capable of binding to human B7H4 comprises a variable heavy chain (VH) region

comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33.

27. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region capable of binding to human B7H4 comprises:

5 a) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 27 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

b) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and

10 CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35;

c) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 37, 38 and 39, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 41, DTS and SEQ ID NO. 42;

d) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively

15 of SEQ ID NOs.: 44, 45 and 46, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 48, YTS and SEQ ID NO. 49;

e) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 51, 52 and 53, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 55, GAS and SEQ ID NO. 56; or

20 f) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 32 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ ID NO. 35

g) a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 66, 67 and 68, and a variable light chain region comprising the CDR1, CDR2 and

25 CDR3 respectively of SEQ ID NO. 70, GAS and SEQ ID NO. 71.

28. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region capable of binding to human B7H4 comprises a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NOs.: 26, 30 and 28, and a variable light chain region comprising the CDR1, CDR2 and CDR3 respectively of SEQ ID NO. 34, GAS and SEQ

30 ID NO. 35.

29. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region capable of binding to human B7H4 comprises:

a) a variable heavy chain (VH) region of SEQ ID NO. 25 : and a variable light chain region of SEQ ID NO. 33;

5 b) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33;

c) a variable heavy chain (VH) region of SEQ ID NO. 36 : and a variable light chain region of SEQ ID NO. 40;

d) a variable heavy chain (VH) region of SEQ ID NO. 43 : and a variable light chain region of SEQ
10 ID NO. 47;

e) a variable heavy chain (VH) region of SEQ ID NO. 50 : and a variable light chain region of SEQ ID NO.54; or

f) a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33

15 g) a variable heavy chain (VH) region of SEQ ID NO. 65 : and a variable light chain region of SEQ ID NO. 69.

30. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen-binding region capable of binding to human B7H4 comprises a variable heavy chain (VH) region of SEQ ID NO. 29 : and a variable light chain region of SEQ ID NO. 33.

20 31. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises

a heavy chain variable region (VH), wherein ÇDR1 comprises the amino acid sequence of SEQ ID NO. 18, wherein CDR2 comprises the amino acid sequence of SEQ ID NO. 19, and
25 wherein CDR3 comprises the amino acid sequence of SEQ ID NO. 21, and

a light chain variable region (VL), wherein CDR1 comprises the amino acid sequence of SEQ ID NO. 23, wherein CDR2 comprises the amino acid sequence of GTN, and wherein CDR3 comprises the amino acid sequence of SEQ ID NO: 24, and

wherein the antigen-binding region capable of binding to B7H4 comprises:

30 a variable heavy chain (VH) region, wherein cpmprises the amino acid sequence of SEQ ID NO. 26, wherein CDR2 comprises the amino acid sequence of SEQ ID NO. 30, and wherein CDR3 comprises the amino acid sequence of SEQ ID NO. 28, and

a light chain variable region (VL), wherein CDR1 comprises the amino acid sequence of SEQ ID NO. 34, wherein CDR2 comprises the amino acid sequence of GAS, and wherein CDR3 comprises the amino acid sequence of SEQ ID NO: 35.

32. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises:

a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO. 17, and, a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 regions of SEQ ID NO: 22. and

wherein the antigen-binding region capable of binding to B7H4 comprises:

a variable heavy chain (VH) region comprising the CDR1, CDR2 and CDR3 regions of SEQ ID NO. 29 : and a variable light chain region comprising the CDR1, CDR2 and CDR3 regions respectively of SEQ ID NO. 33.

33. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein the antigen-binding region that binds to CD3 comprises a variable heavy chain (VH) region of SEQ ID NO: 17 and a variable light chain region of SEQ ID NO: 22 and wherein the antigen-binding region capable of binding to human B7H4 comprises a variable heavy chain (VH) region of SEQ ID NO: 29 : and a variable light chain region of SEQ ID NO: 33.

34. A pharmaceutical composition according to any one of the preceding claims, wherein

the antigen-binding region capable of binding to B7H4 is human, and/or

the antigen-binding region capable of binding to CD3, is humanized.

35. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises two heavy chain constant regions (CH), and two light chain constant regions (CL), preferably wherein the two heavy chain constant domains, and two light chain constant regions are derived from human.

36. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody is a full-length antibody.

37. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody is of the IgG1 isotype.

38. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises a first and a second heavy chain, each of said first and second heavy chain comprises at

least a hinge region, a CH2 and CH3 region, wherein in said first heavy chain at least one of the amino acids in the positions corresponding to positions selected from the group consisting of T366, L368, K370, D399, F405, Y407 and K409 in a human IgG1 heavy chain has been substituted, and in said second heavy chain at least one of the amino acids in the positions corresponding to a position
5 selected from the group consisting of T366, L368, K370, D399, F405, Y407, and K409 in a human IgG1 heavy chain has been substituted, wherein said substitutions of said first and said second heavy chains are not in the same positions, and wherein the amino acid positions are numbered according to Eu numbering.

39. A pharmaceutical composition according to claim 38, wherein the amino acid in the position
10 corresponding to K409 in a human IgG1 heavy chain is R in said first heavy chain, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain is L in said second heavy chain, or *vice versa*.

40. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises a first and, optionally, a second heavy chain and wherein the first heavy chain, and the
15 second heavy chain if present, is/are modified so that the antibody induces Fc-mediated effector function to a lesser extent relative to an identical non-modified antibody.

41. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises a first and a second heavy chain, and wherein in both the first and the second heavy chain, the amino acid residues at the positions corresponding to positions L234 and L235 in a human IgG1
20 heavy chain according to Eu numbering are F and E, respectively.

42. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises a first and a second heavy chain, and wherein in both the first and the second heavy chain, the amino acid residue at the position corresponding to position D265 in a human IgG1 heavy chain according to Eu numbering is A.

25 43. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody comprises a lambda (λ) light chain and a kappa (κ) light chain; e.g. an antibody with a heavy chain and a lambda light chain which comprise the binding region capable of binding to CD3, and a heavy chain and a kappa light chain which comprise the binding region capable of binding to B7H4.

44. A pharmaceutical composition according to any one of the preceding claims, wherein the antigen
30 binding region capable of binding to human B7H4 is comprised in an heavy chain and a light chain, said heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a kappa light chain constant region; and wherein said antigen binding region capable of binding to human CD3 is comprised in a heavy chain and a light chain, said

heavy chain comprising said VH region and an IgG1 heavy chain constant region and said light chain comprising said VL region and a lambda light chain constant region.

45. A pharmaceutical composition according to claim 44, wherein one IgG1 heavy chain constant region is as defined in SEQ ID NO. 60 and the other is as defined in SEQ ID NO. 61, and wherein said kappa light chain constant region is as defined in SEQ ID NO. 63 and said lambda light chain constant region is as defined in SEQ ID NO. 64.

46. A pharmaceutical composition according to claim 45, wherein said IgG1 heavy chain constant regions as defined in SEQ ID NO. 60 and 61 have their terminal lysines deleted.

47. A pharmaceutical composition according to any one of the preceding claims, wherein the antibody is bsIgG1-huCD3-H101G-FEALxB7H4-C1-N52S-FEAR, or a biosimilar thereof.

48. The pharmaceutical composition as defined in any of claims 1 to 47 for use as a medicament.

49. The pharmaceutical composition for use as a medicament according to claim 48 for use in a method for the treatment of a disease.

50. The pharmaceutical composition for use according to claim 49, wherein the disease is cancer, optionally wherein the cancer is characterized by expression of B7H4 in cancer cells, optionally wherein the cancer is a solid tumor.

51. The pharmaceutical composition for use according to claim 50, wherein the cancer is selected from the group consisting of lung cancer, NSCLC (ADC or SQCC), stomach cancer, pancreas cancer, cholangiocarcinoma, bladder cancer, cervical cancer, head and neck cancer, breast cancer, ovarian cancer and uterine cancer.

52. A method of treating a disease, the method comprising administering a pharmaceutical composition as defined in any one of claims 1-47 to a subject in need thereof.

53. A method of treating cancer in a subject comprising administering to a subject in need thereof the pharmaceutical composition of any one of claims 1 to 47 for a time sufficient to treat the cancer.

54. Use of the pharmaceutical composition of any one of claims 1-47 for the treatment of cancer.

55. A unit dosage form, comprising:

a) an antibody comprising an antigen-binding region capable of binding to human B7H4 and an antigen-binding region capable of binding to human CD3, wherein said antigen-binding regions comprise heavy and light chain variable regions, wherein said heavy and light chain variable regions are humanized and/or human, in an amount of from 5 pg to 1200 mg,

and

b) a buffering agent, preferably selected from the group consisting of histidine, glutamate, and mixtures thereof,

wherein the pH of the unit dosage form is from 4.0 to 8.0, preferably 4.5 to 6.5, more preferably 5.0 to 6.0.

56. A unit dosage form of claim 55, wherein the antibody is as defined in any one of claims 20-47.

57. A unit dosage form of claims 55 or 56, wherein the amount of the antibody is from 20 mg to 1000 mg, such as 200 mg to 600 mg, such as 300 mg to 400 mg.

58. A kit-of-parts comprising:

a. the pharmaceutical composition of any one of claims 1-47, or the unit dosage form of any of claims 55-57,

b. a receptacle for the pharmaceutical composition or for the unit dosage form

c. directions for dilution and/or for use.

59. A method of preparing a pharmaceutical composition as defined in any one of claims 1-47, comprising the steps of mixing in water for injection:

a. 0.5 to 120 mg/ml of the antibody, and

b. a buffering agent

and adjusting the pH to 4.0-8.0, preferably to 5.0-6.0.

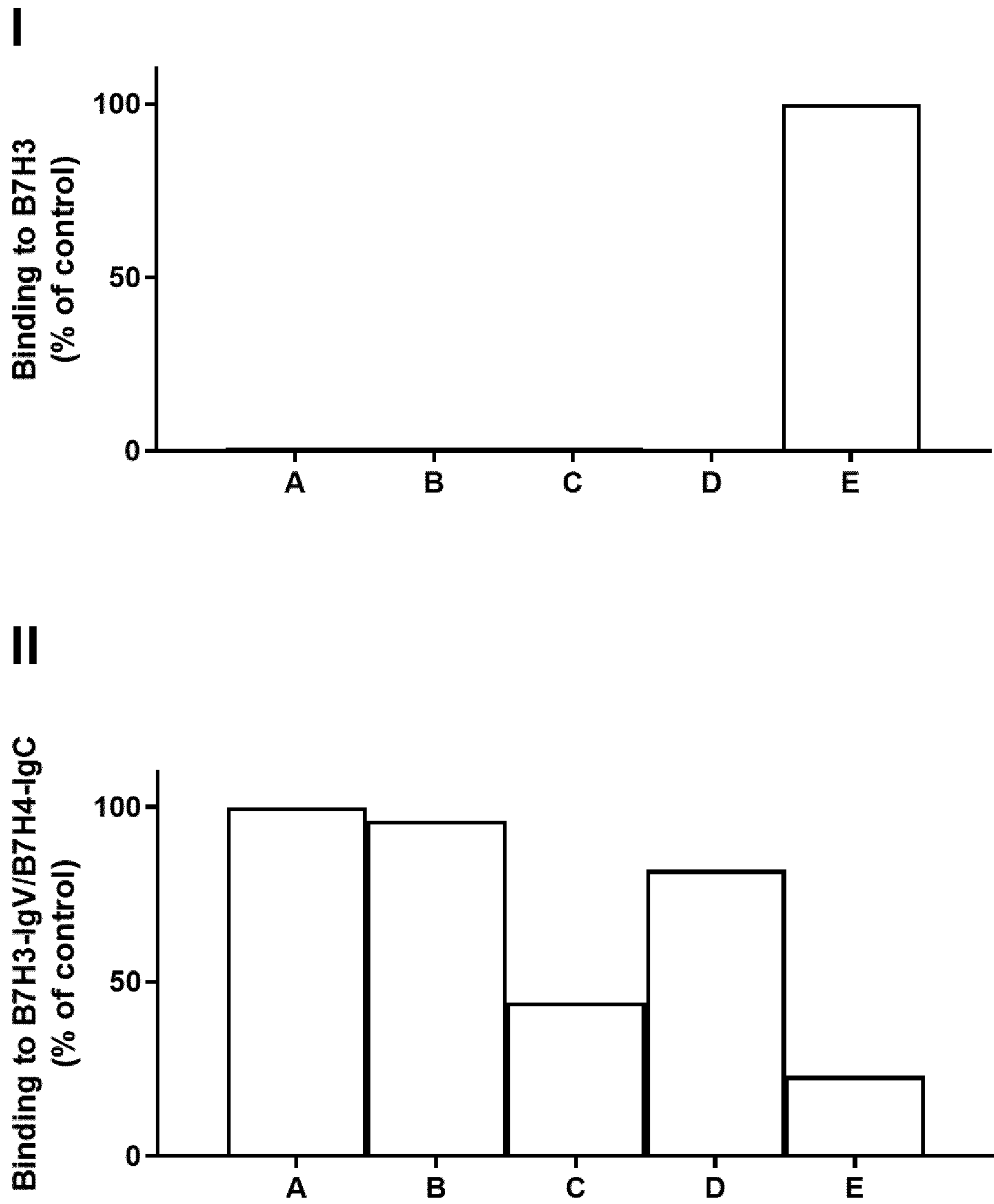
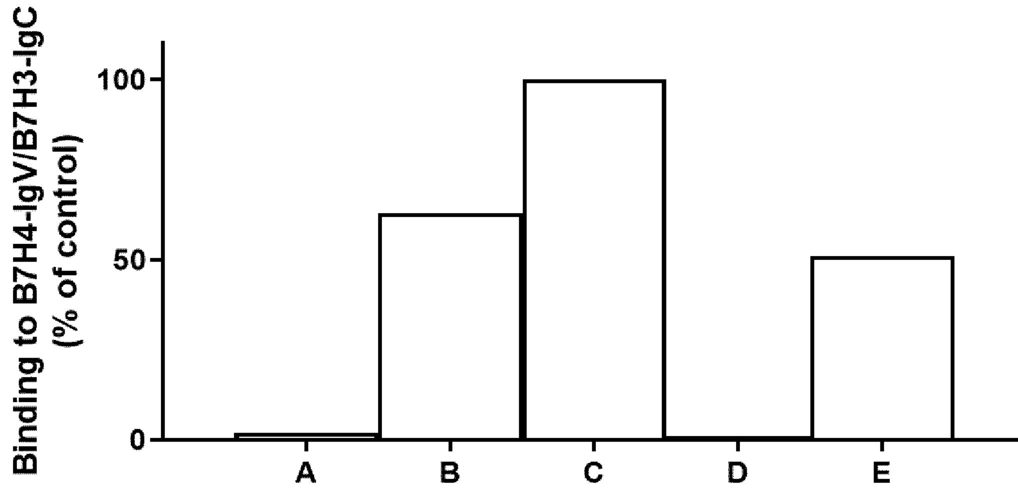


Figure 1



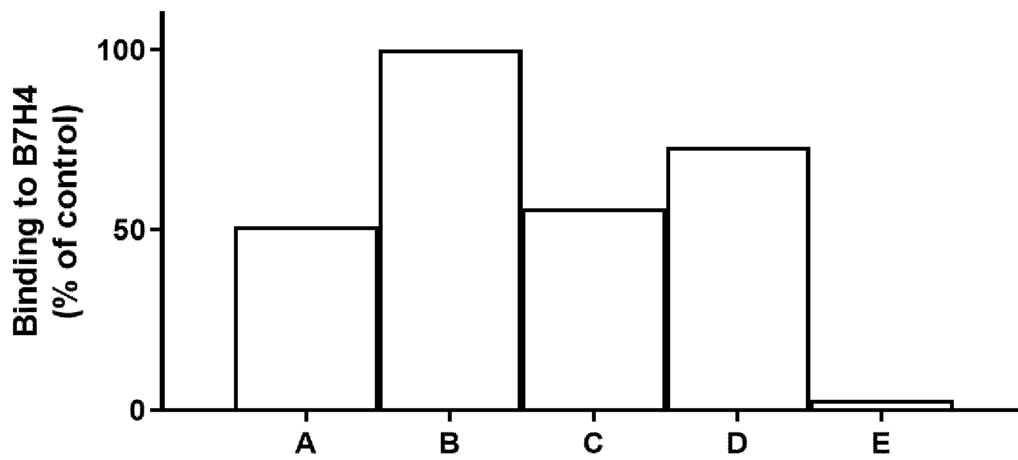
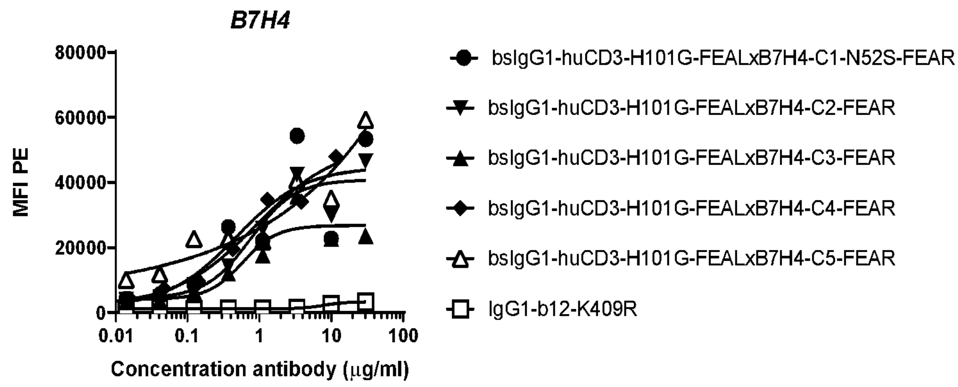
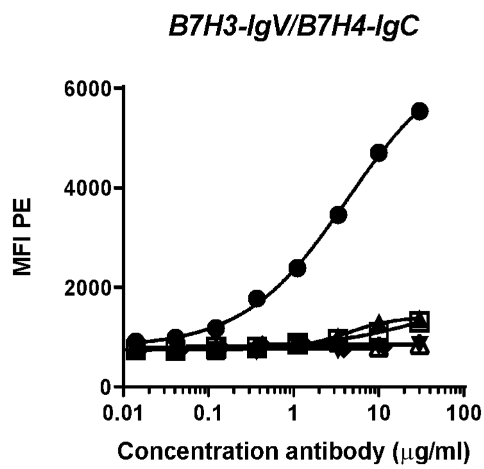


Figure 1 (continued)





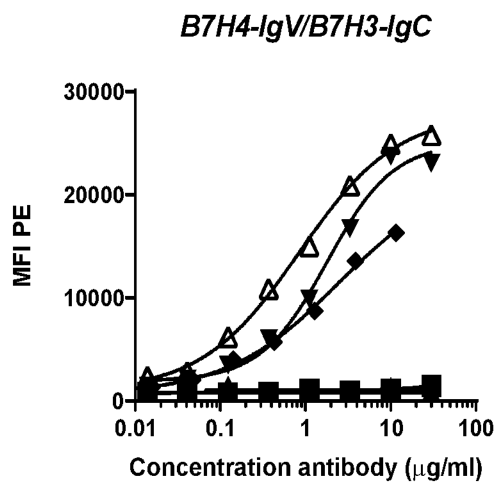


Figure 2

A

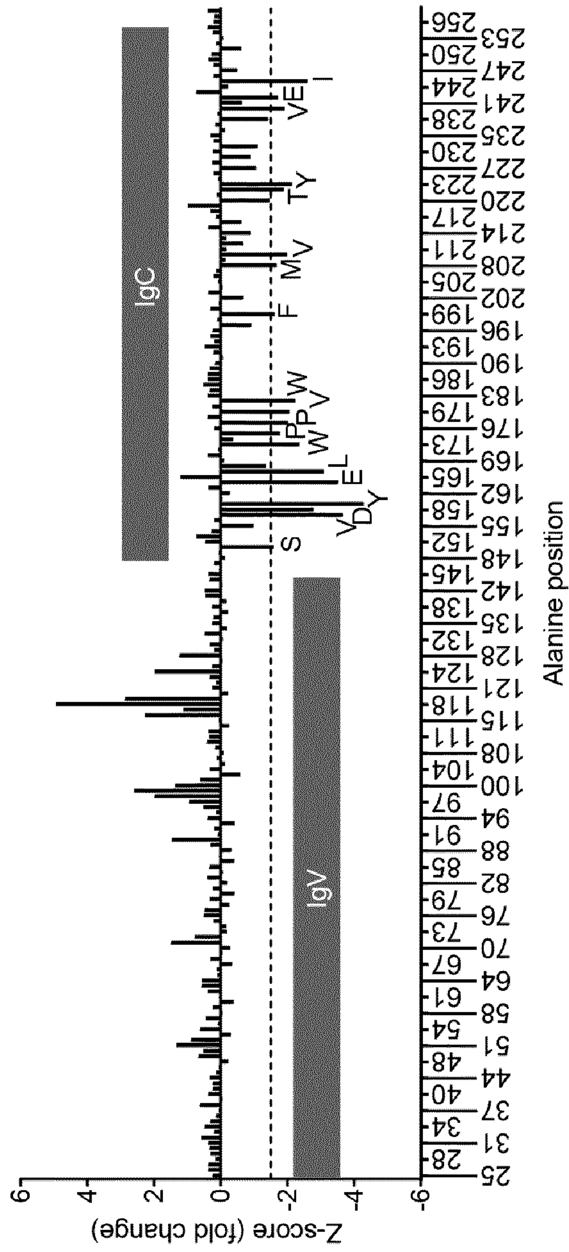


Figure 3

B

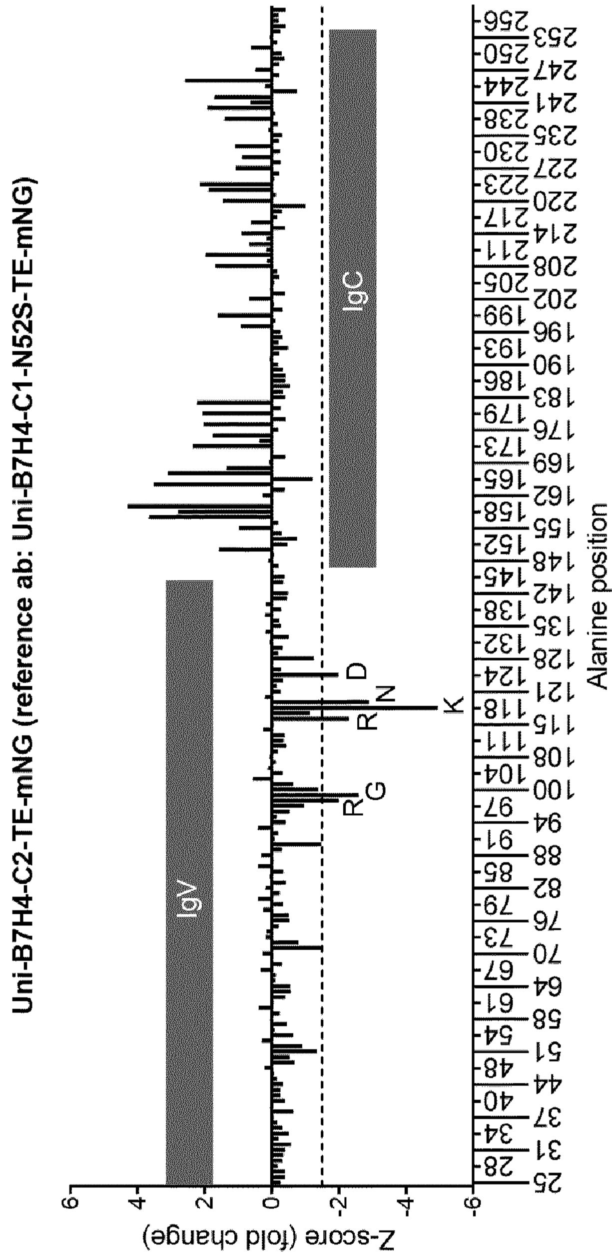


Figure 3 (continued)

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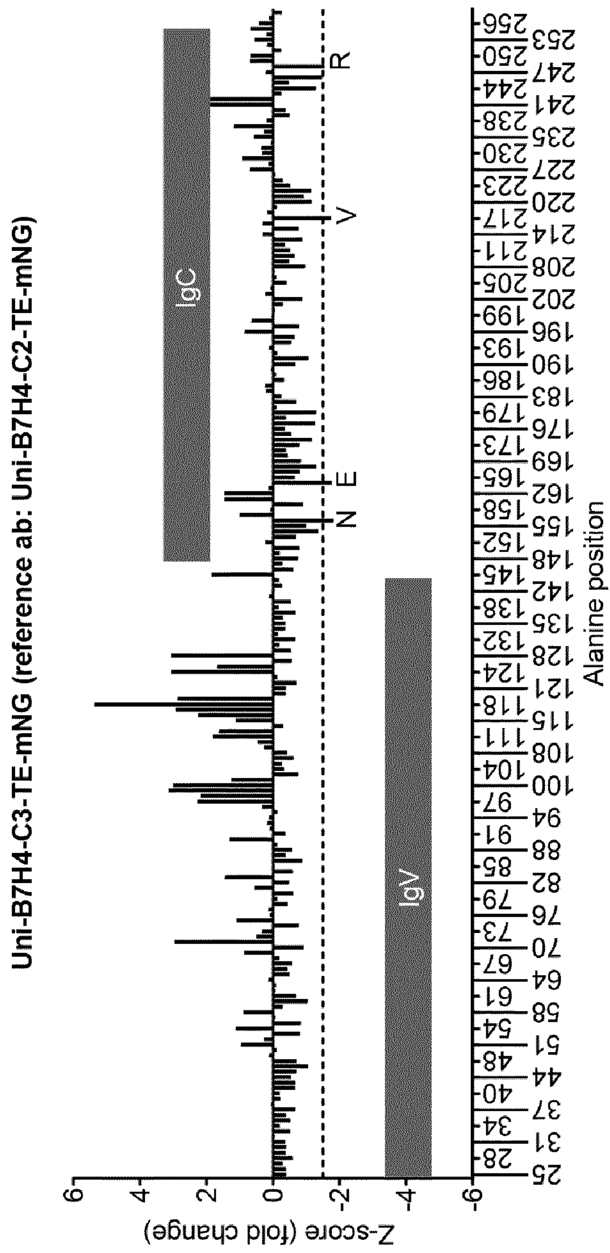

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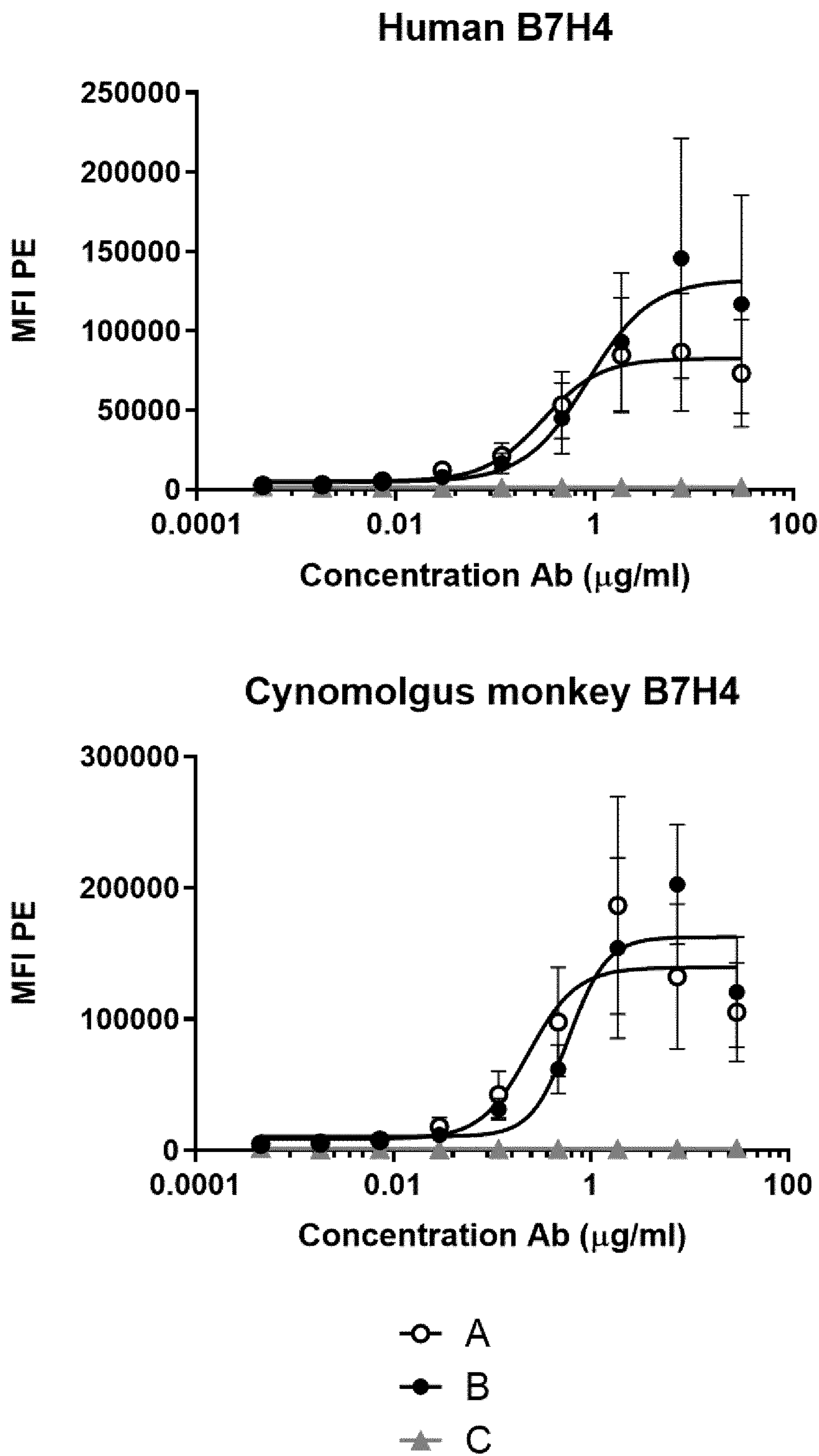

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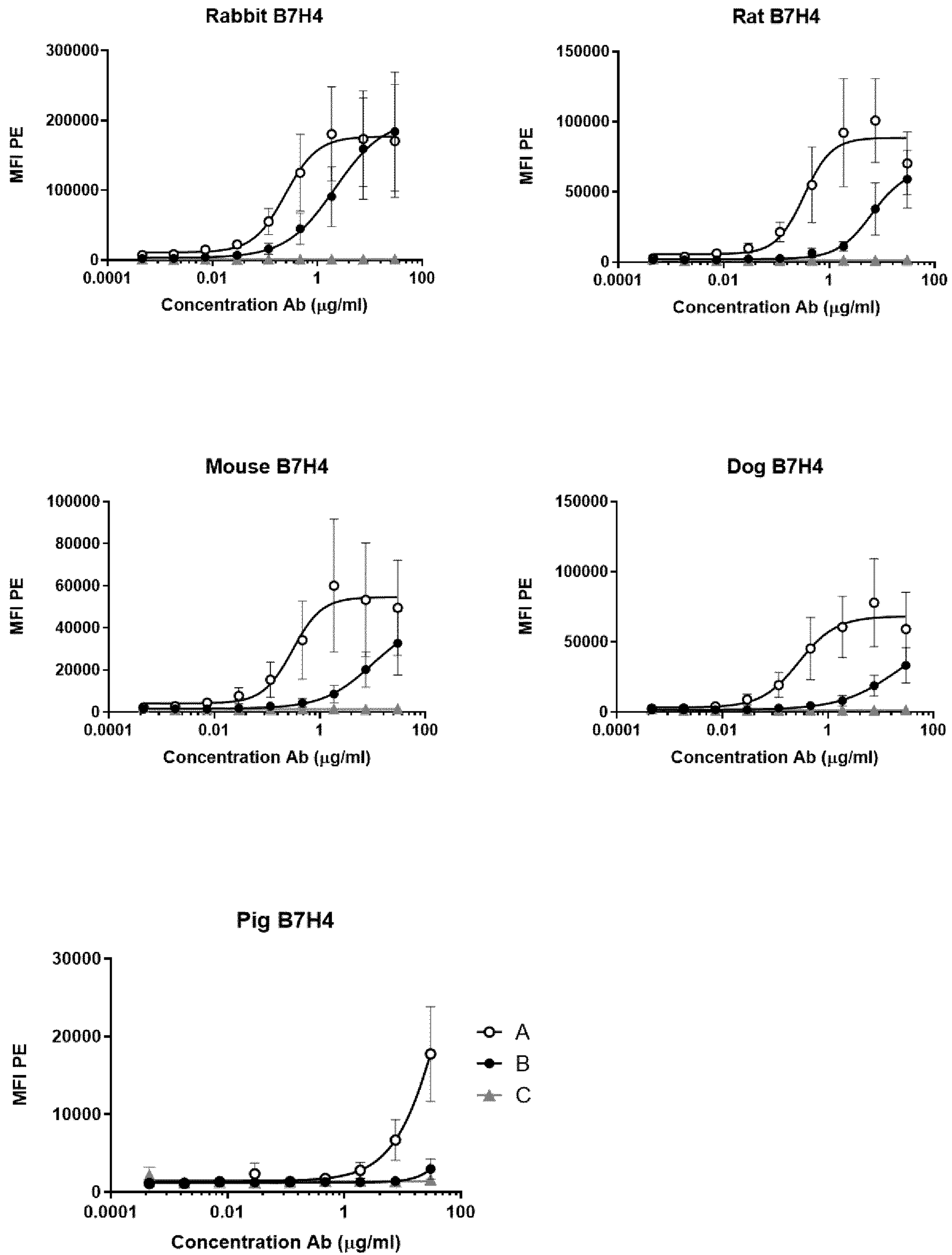

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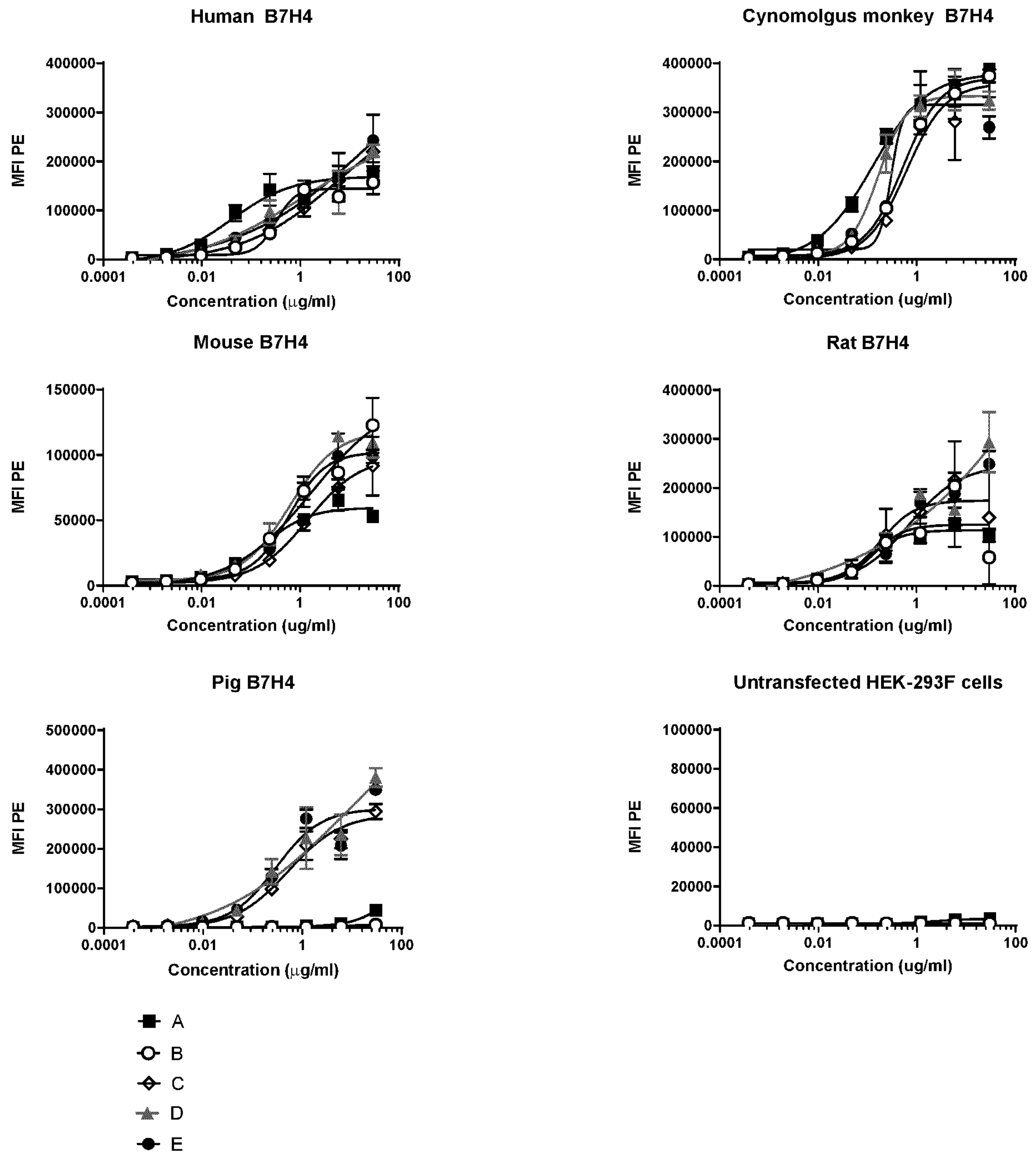

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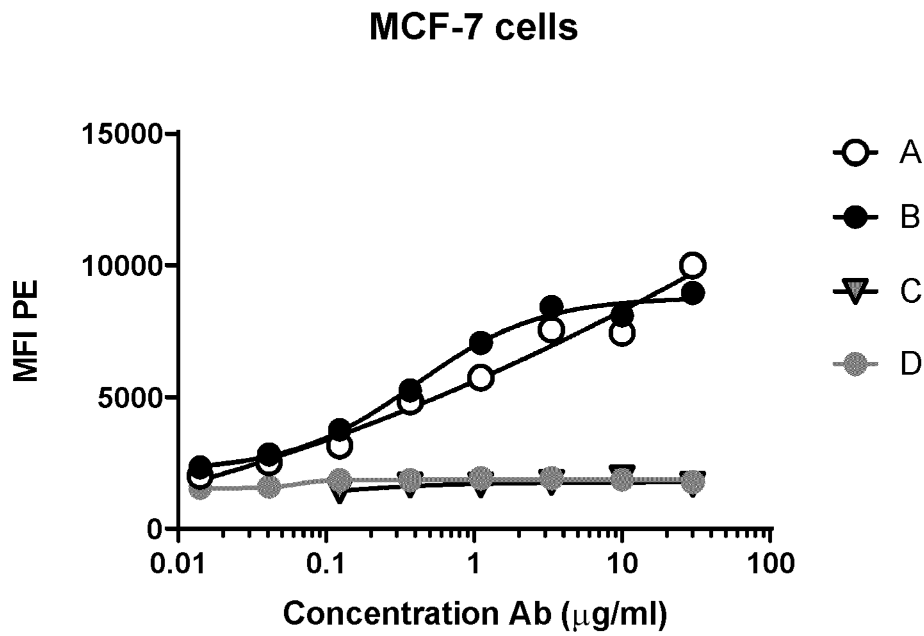

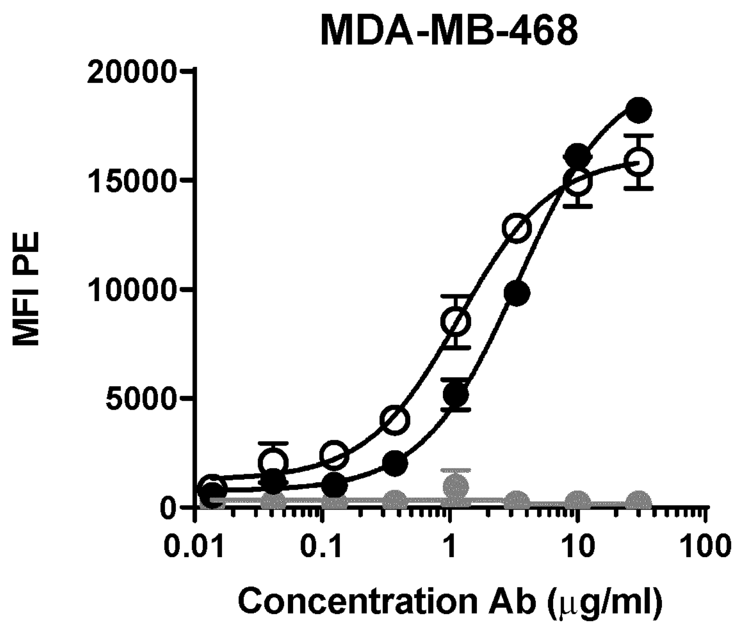

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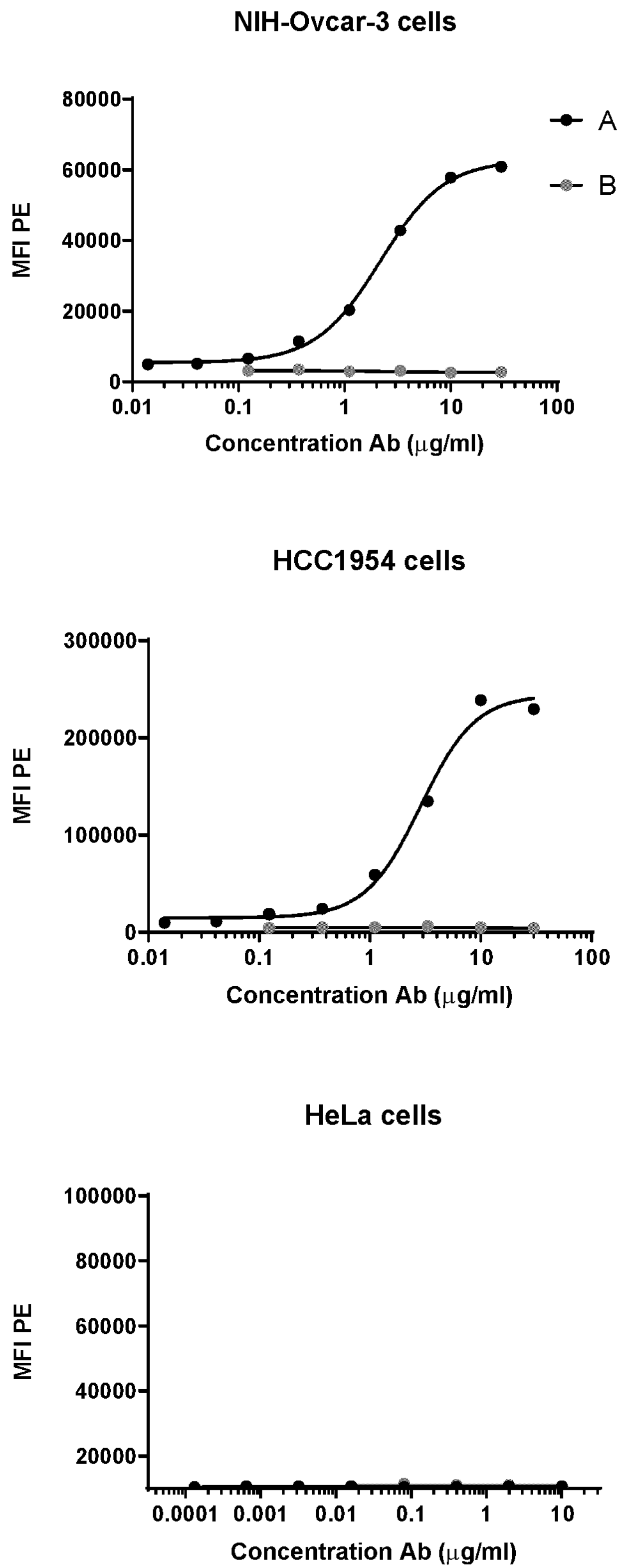

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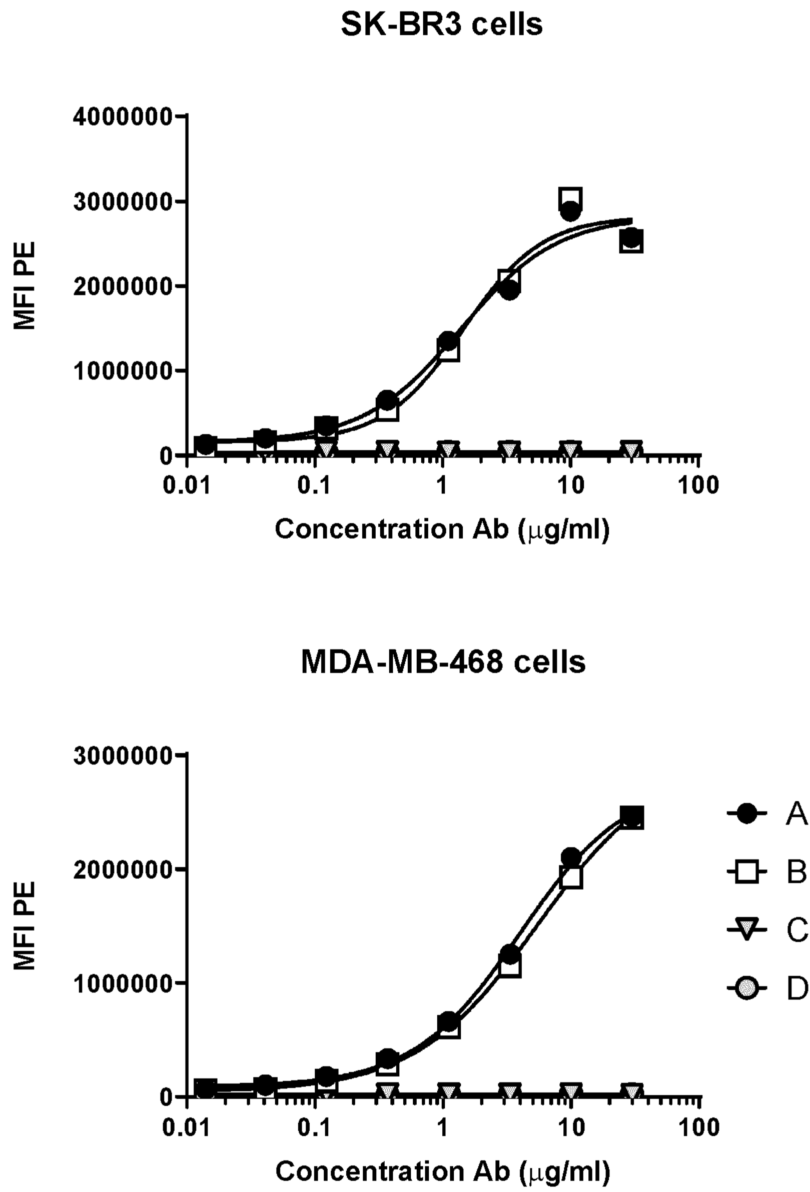
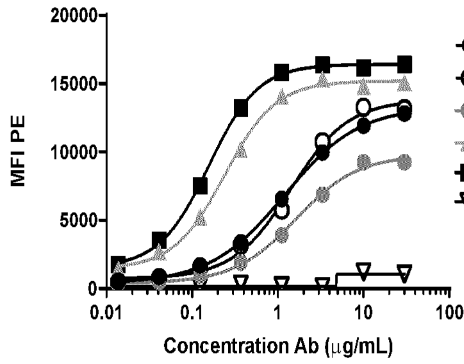
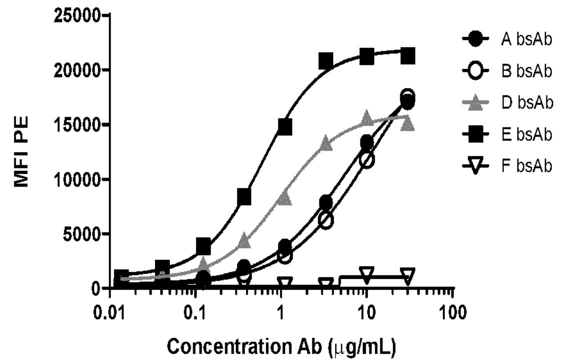


Figure 9

MDA-MB-468





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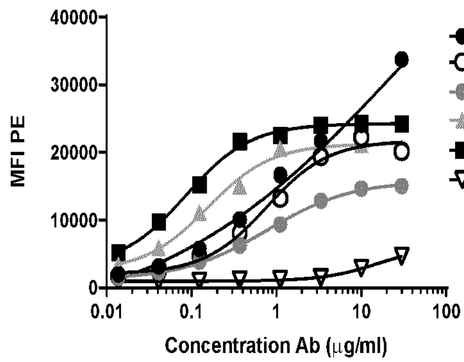


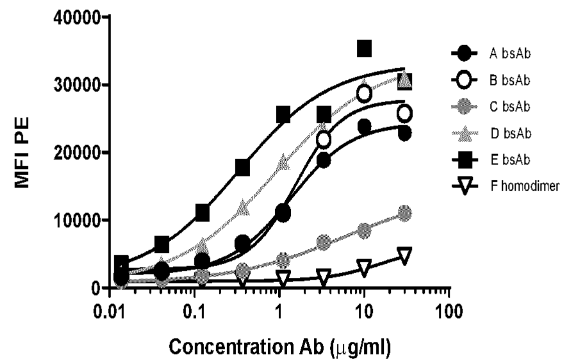
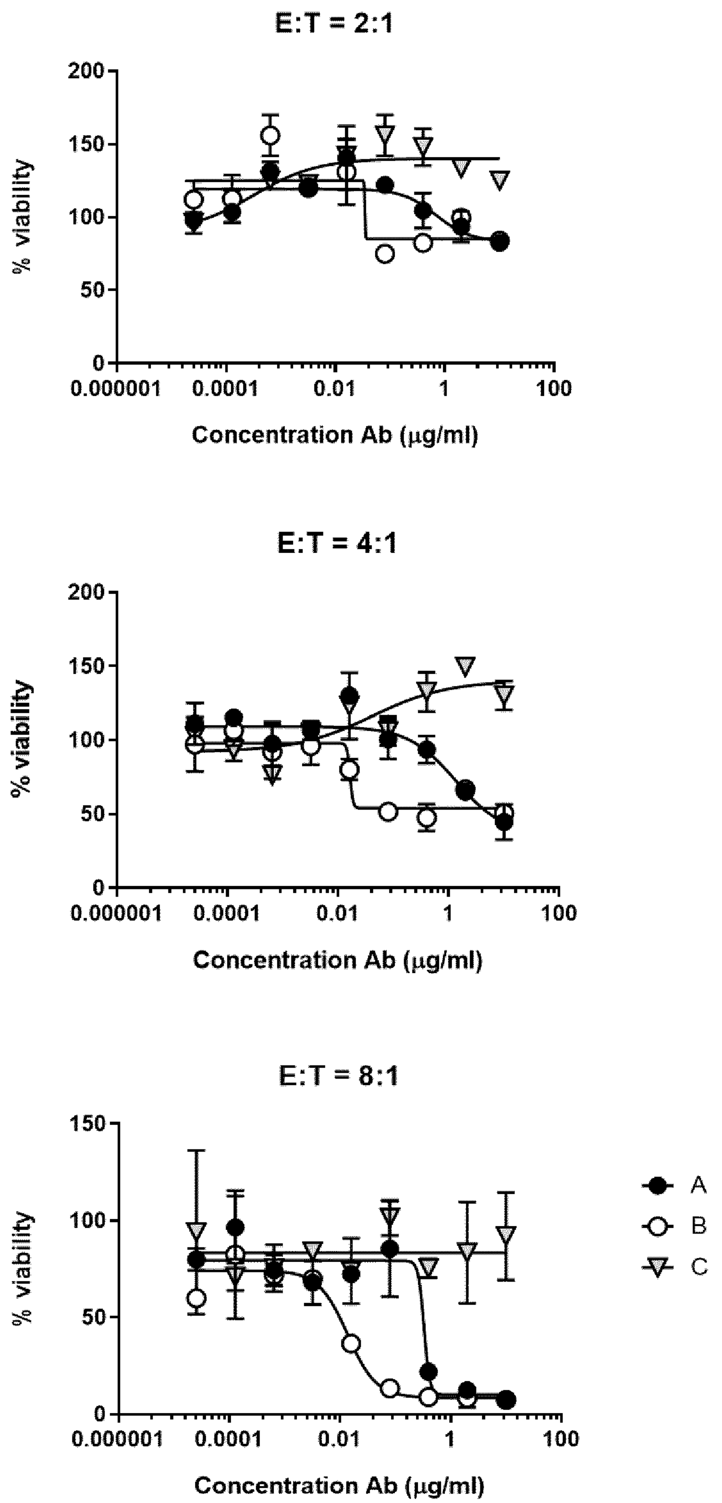


Figure 10



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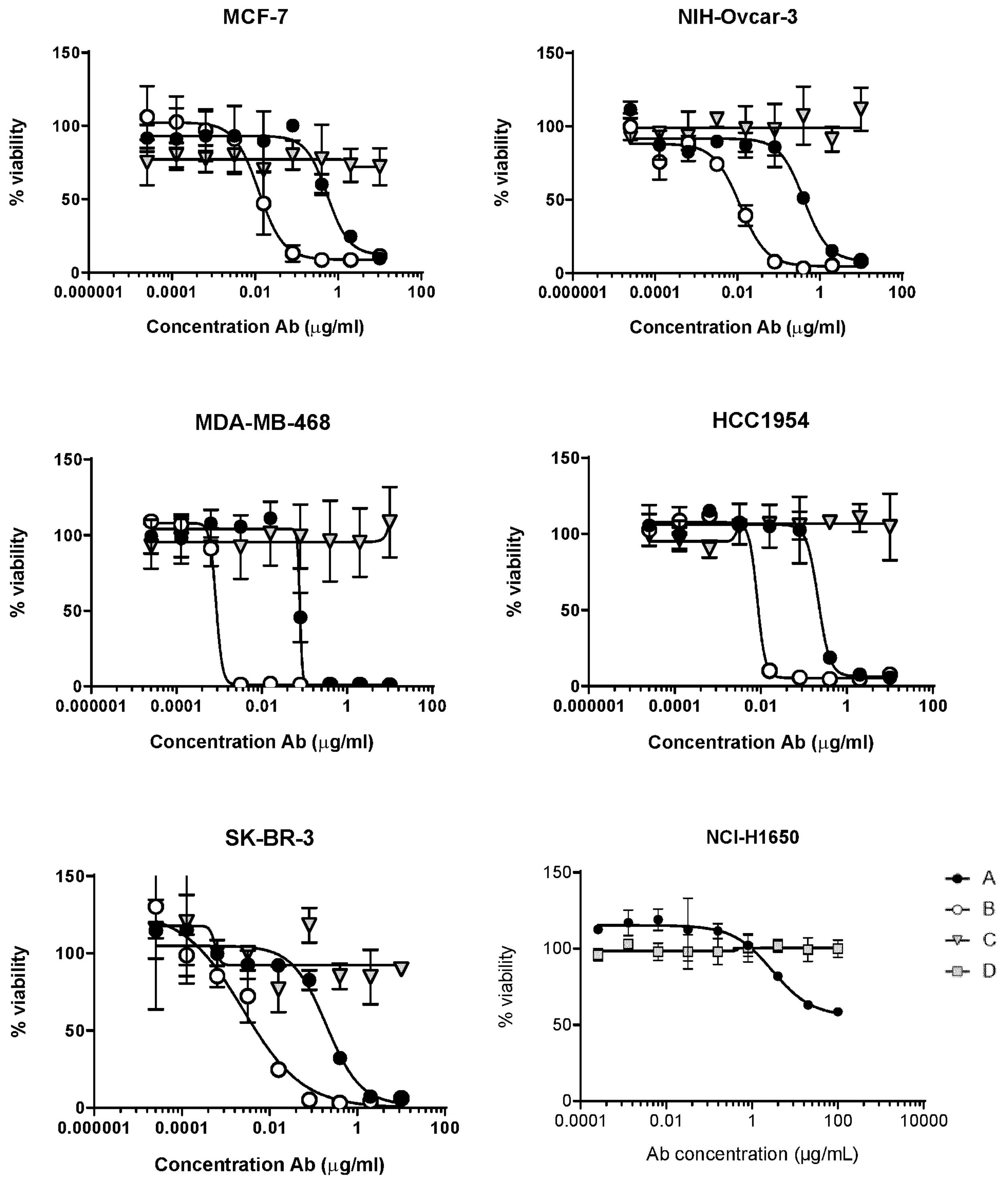

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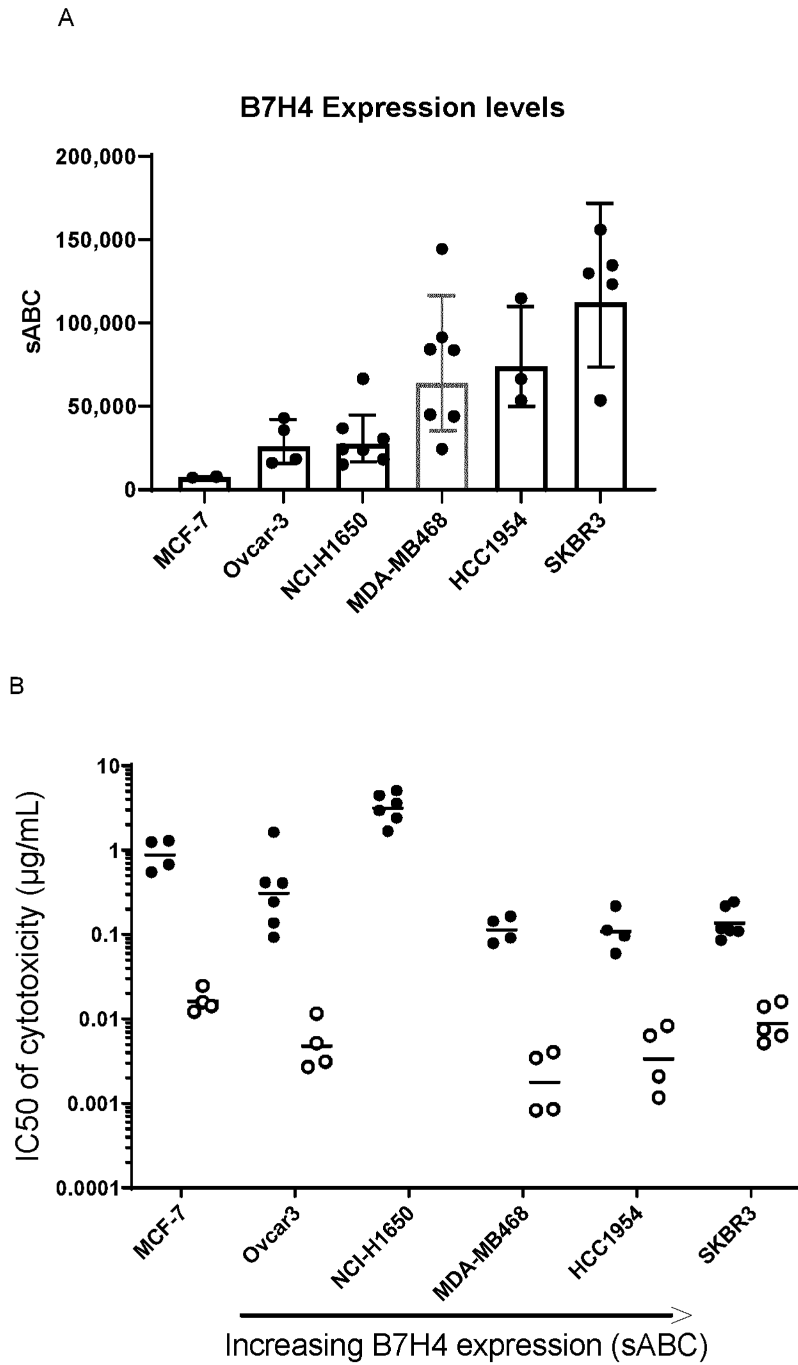

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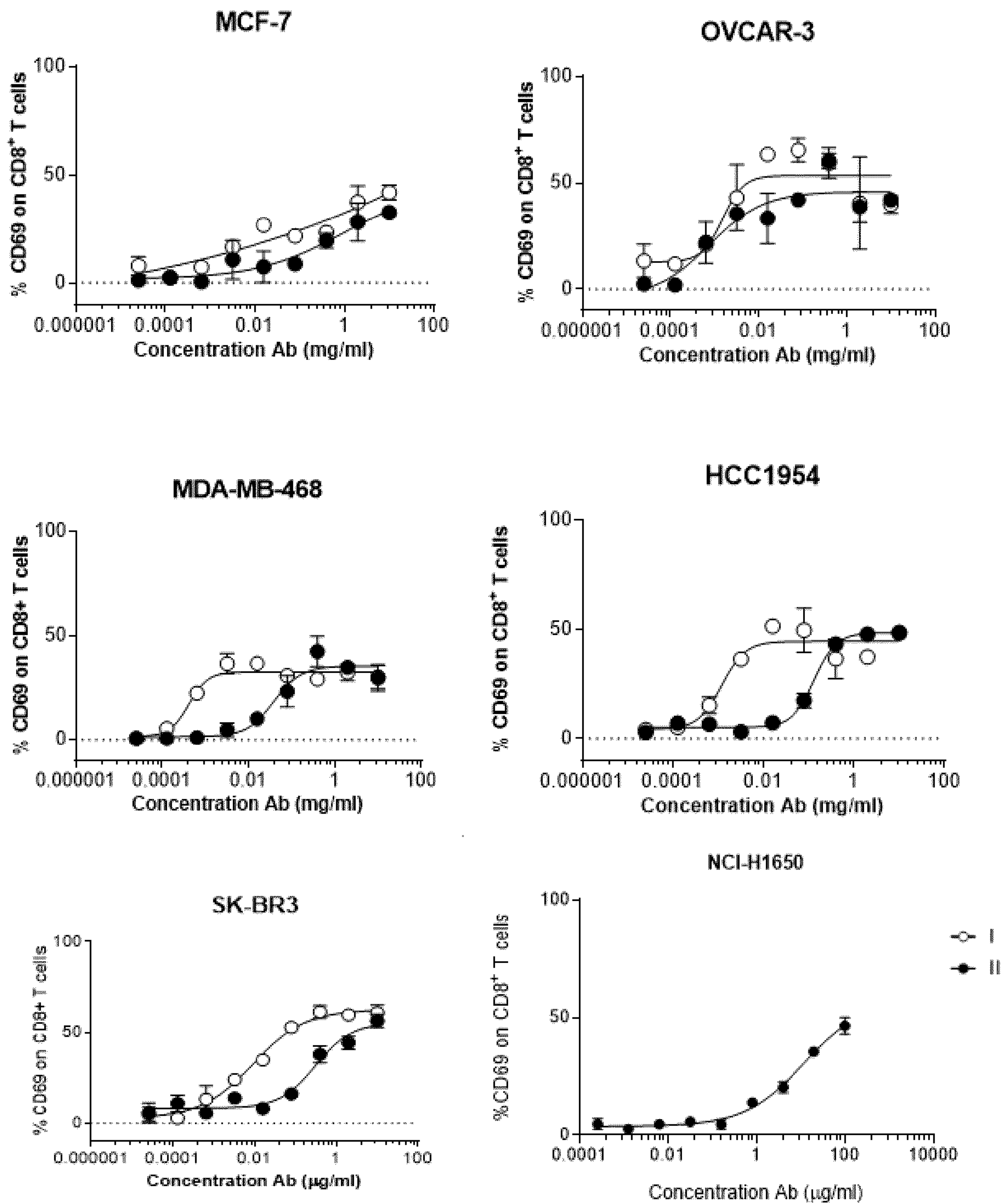
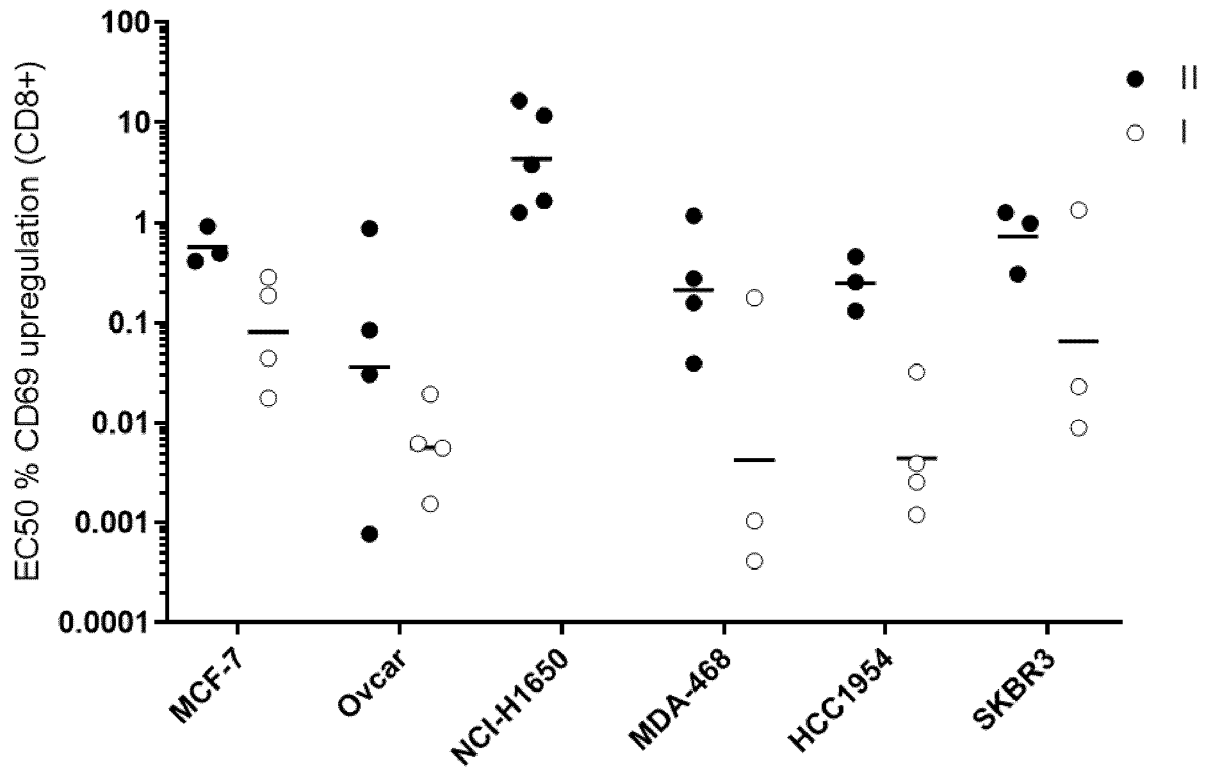


Figure 14

B.



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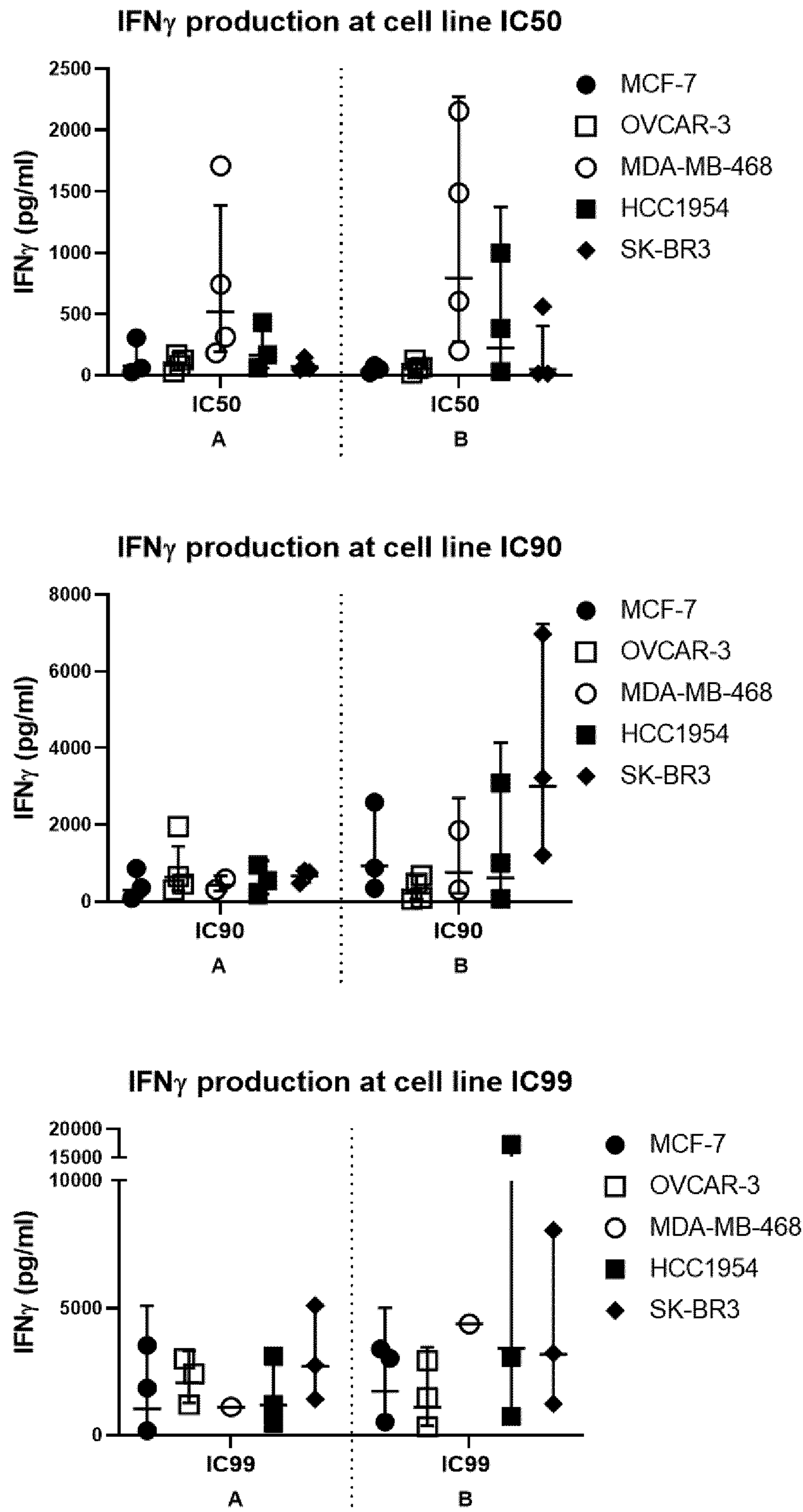

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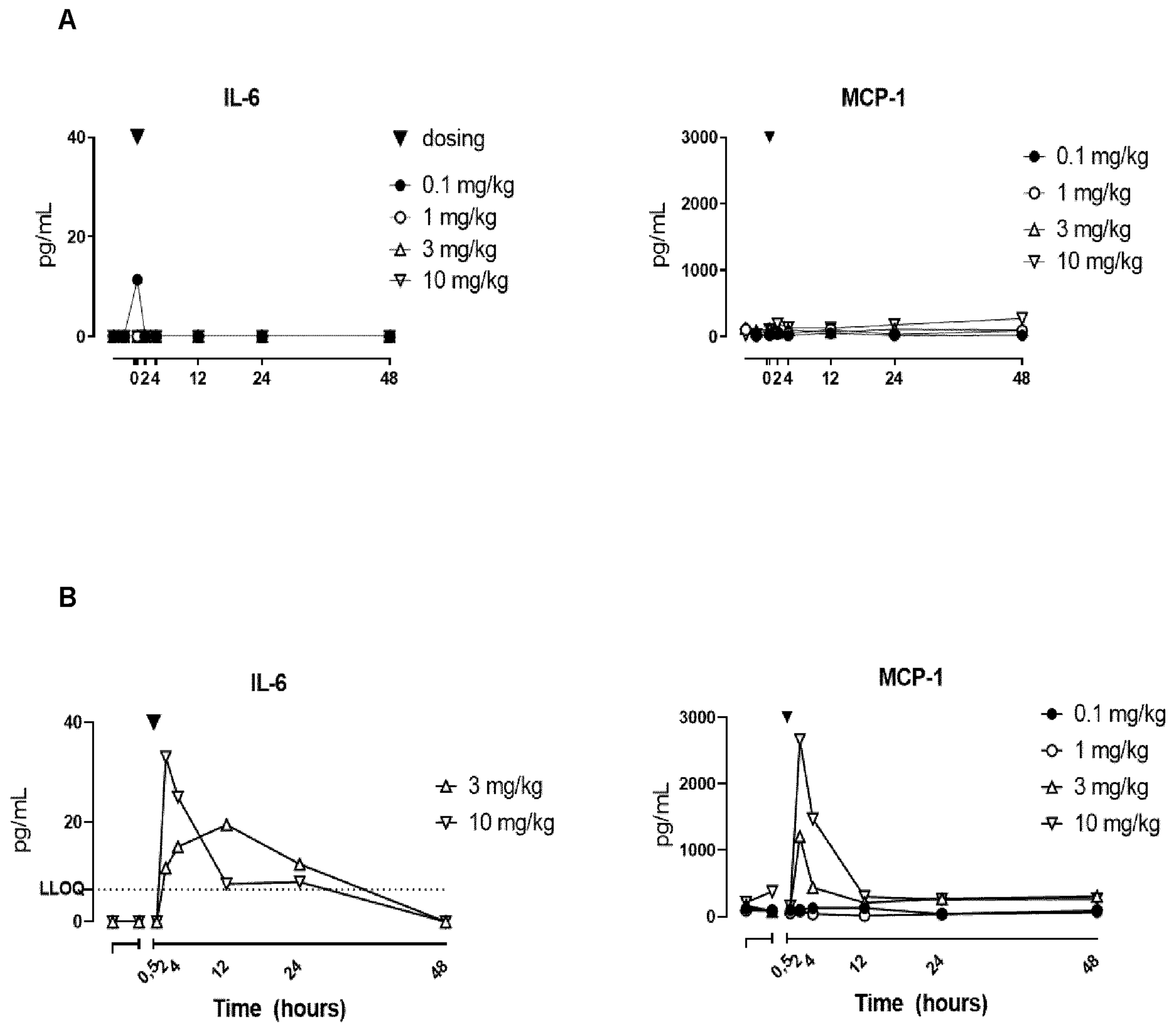
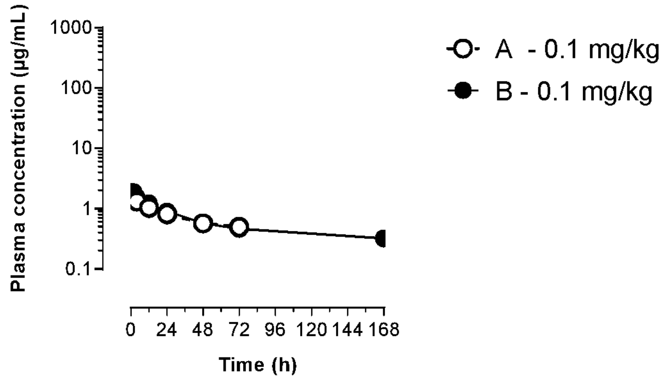
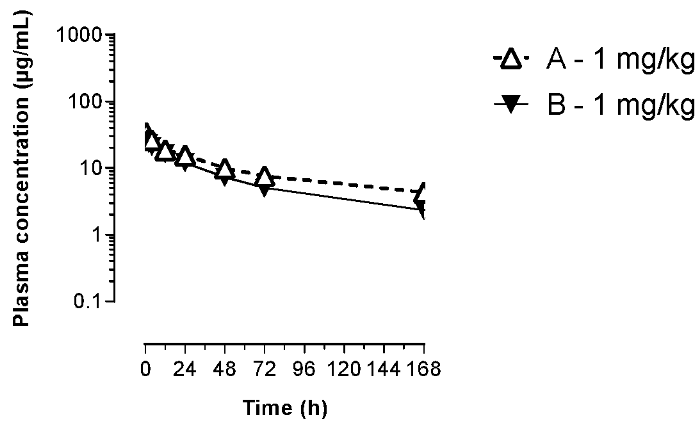
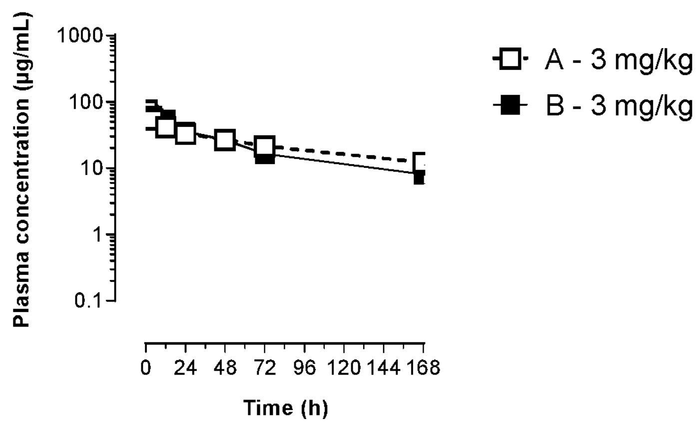


Figure 16







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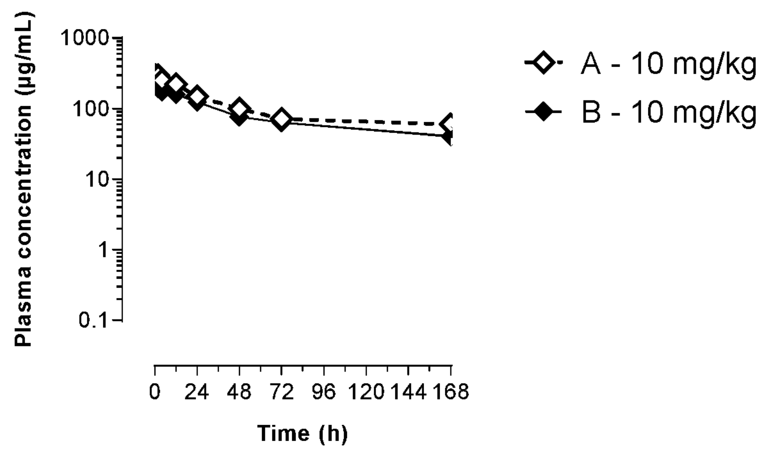

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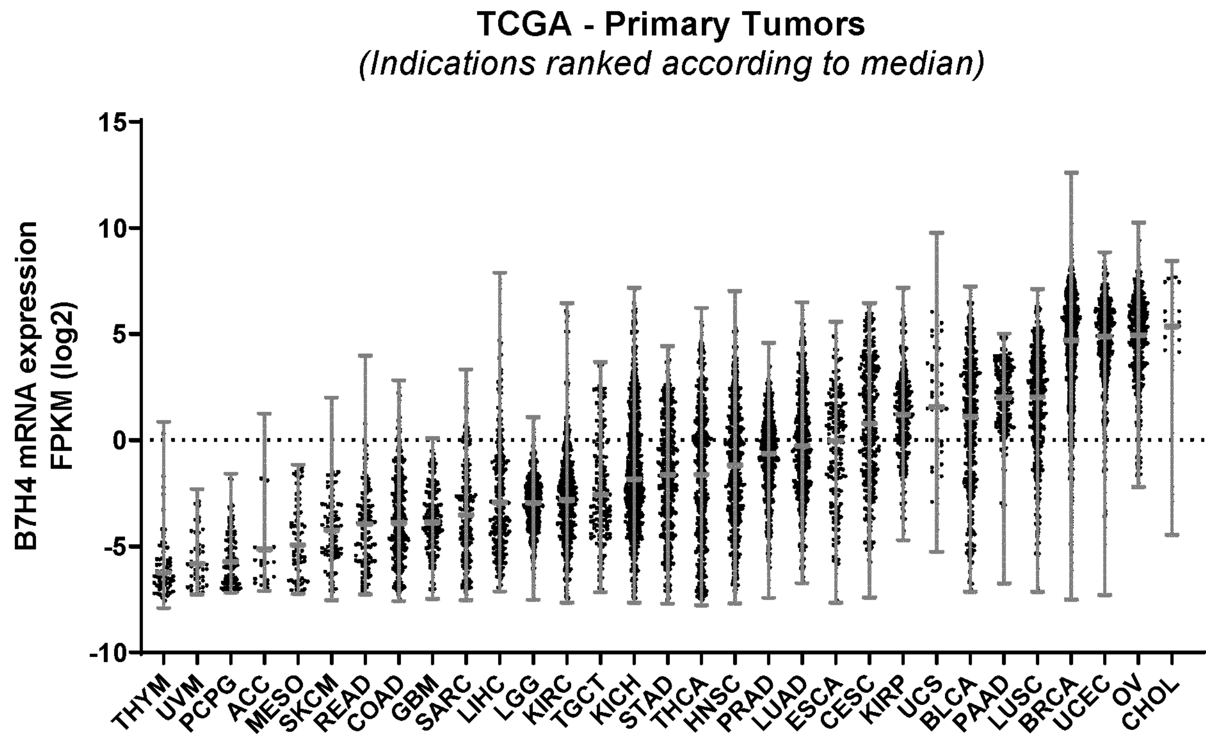

Figure 18