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(54) Title: ANTIBODIES BINDING TO CD30 AND CD3

(57) Abstract: The invention relates to multispecific antibodies binding to CD3 and CD30. The invention further provides pharmaceutical compositions comprising antibodies of the invention, nucleic acids encoding the antibodies, host cells that produce the antibodies, methods of producing the antibodies and uses of the antibodies, in particular for cancer therapy.

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ANTIBODIES BINDING TO CD30 AND CD3

FIELD OF THE INVENTION

The present invention relates to multispecific antibodies binding to CD30 and CD3. The invention further provides pharmaceutical compositions comprising the antibodies, nucleic acids encoding the antibodies, host cells that produce the antibodies, methods of producing the antibodies and uses of the antibodies, in particular for cancer therapy.

BACKGROUND OF THE INVENTION

CD30, also known as Ki-1 or TNFRSF8, is a 120 kD transmembrane glycoprotein receptor and is a member of the tumor necrosis factor receptor (TNFR) superfamily (Smith et al. (1994) Cell 76: 959–962). CD30 is a single-pass type I membrane protein with six cysteine-rich repeats in its extracellular domain (Durkop et al. (1992) Cell 68: 421–427). In addition, a soluble form of CD30 (sCD30) has been detected in the serum of patients with inflammatory diseases, including ulcerative colitis (UC) (Giacomelli et al. Clin Exp Immunol. 1998;111:532–5), and CD30-positive hematologic malignancies (Josimovic-Alasevic et al. (1989) Eur J Immunol). sCD30 represents the cleavage product of the extracellular portion of CD30 by cell membrane-anchored metalloproteinases such as TACE/ADAM17 and ADAM10 (Nagata et al., PNAS 2005; Hansen et al., FASEB, 2004).

In normal tissues CD30 expression is mainly restricted to subsets of activated T and B lymphocytes (Bowen et al. (1996) J Immunol. 156:442–9; Shanebeck et al. (1995) Eur J Immunol. 25:2147–53). CD30 expression has been detected in a variety of lymphoid neoplasms. Classical Hodgkin lymphoma (cHL) and anaplastic large cell lymphoma (ALCL) show high CD30 expression levels. In particular, the large majority of Reed–Sternberg (RS) cells, which are typically found in cHL, are positive for CD30 (Frizzera et al. (1992) Semin. Diagn. Pathol. 9: 291–296).

For example, the CD30-targeting antibody-drug conjugate brentuximab vedotin (BV; SGN-35) has been used for or suggested for use in treating cancers such as cHL, ALCL and CTCL (Younes et al. J Clin Oncol. 2012 Jun 20;30(18):2183-9; Pro et al. Blood. 2017 Dec 21;130(25):2709-2717; Shea et al. Curr Hematol Malig Rep. 2020 Feb;15(1):9-19). The tetravalent bispecific CD30xCD16A antibody AFM13 is being developed as NK cell-mediated immunotherapy for cHL and CD30-positive lymphoma (Rothe et al. Blood. 2015 Jun 25;125(26):4024-31).

Furthermore, CD30-targeting CAR-T cell therapies are being developed for cHL and CD30-positive lymphoma. Other CD30 antibodies have been described in WO2003059282 (Medarex),

US8257706 (Seattle genetics), US20100239571 (Seattle genetics) WO2007040653 (US government & Health), and WO20160177846 (Affimed).

Pohl et al. (1993 Int. J. Cancer, 54: 820-827) describe the CD3xCD30 bispecific antibody OKT-3/HRS-3 that was generated by fusion of the CD30 monoclonal antibody HRS-3-producing hybridoma cells with CD3 monoclonal antibody OKT-3-producing hybridoma cells (hybrid hybridoma technology).

WO2008119567 describes the generation and characterization of CD30 and CD3 cross-species specific bispecific single chain molecules.

US20200095330 describes CD3xCD30 bispecific antibodies resulting from the chemical heteroconjugation of two anti-CD30 clones (named 8D10 and 10C2) to anti-CD3 (Orthoclone OKT-3).

However, none of these CD3xCD30 bispecific antibodies have been tested in a clinical setting.

There is therefore a need for improved CD30-targeting cancer therapy. There is a need for CD30 targeting compounds that are efficacious, safe, have good manufacturability and/or have a long shelf-life.

SUMMARY OF THE INVENTION

The present invention relates to T-cell engaging antibodies that bind human and cynomolgus monkey CD30 and CD3. The inventors have found that some CD30 binding antibodies bind well to CD30-expressing cells in a bivalent (monoclonal) format but exhibit strongly reduced binding and cytotoxicity in a bispecific format with monovalent CD30 binding. Bispecific antibodies having strong monovalent CD30 binding as well as excellent tumor cell killing were identified. Furthermore, a bispecific antibody with a functionally inactive Fc backbone was identified which is suitable for development into a pharmaceutical product, because of excellent stability and solubility.

In one aspect, the invention relates to a multispecific antibody comprising:

(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively.

In a further aspect, the invention relates to a nucleic acid construct, or a combination of nucleic acid constructs, encoding a multispecific antibody according to the invention.

In further aspects, the invention relates to an expression vector or a delivery vehicle comprising a nucleic acid construct(s) according to the invention.

In a further aspect, the invention relates to a recombinant host cell capable of producing the multispecific antibody according to the invention, wherein the host cell comprises one or more nucleic acid constructs encoding the multispecific antibody according to the invention.

In a further aspect, the invention relates to a pharmaceutical composition comprising a multispecific antibody according to the invention and a pharmaceutically-acceptable carrier.

In further aspects, the invention relates to multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition according to the invention for use as a medicament, for example in the treatment of cancer.

In even further aspects, the invention relates to methods for producing multispecific antibodies according to the invention.

DESCRIPTION OF THE FIGURES

Figure 1: Binding of bispecific CD3xCD30 antibodies and their monospecific, bivalent CD3 and CD30 counterparts to SU-DHL-1 or HDML-2 cells. Dose-dependent binding of (A) bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-FEALxb12-FEAR, bsG1-b12-FEALxCD30-MDX060-FEAR, and IgG1-CD30-MDX060-FEAR, (B) bsG1-huCD3-FEALxCD30-hAC10-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-hAC10-FEAR, (C) bsG1-huCD3-FEALxCD30-HRS-3-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-HRS-3-FEAR, (D) BsIgG1-huCD3-FEALxCD30-HeFi-I-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-HeFi-I-FEAR, (E) bsIgG1-huCD3-FEALxCD30-T405-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-T405-FEAR, (F) bsIgG1-huCD3-FEALxCD30-T105-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-T105-FEAR, (G) bsIgG1-huCD3-FEALxCD30-T408-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-T408-FEAR, and (H) bsIgG1-huCD3-FEALxCD30-T215-FEAR, bsG1-huCD3-FEALxb12-FEAR, and IgG1-CD30-HRS-3-FEAR to SU-DHL-1 cells (left panels) or HDLM-2 cells (right panels). (I) Binding of CD3xCD30 bispecific antibodies and CD30 monospecific antibodies to SU-DHL-1 (left panels) or HDLM-2 cells (right panels) at a concentration of 1.11 µg/mL. The antibody clones used for the CD30 arm are indicated on the x-axis. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 2: Binding of bsG1-huCD3xCD30-MDX060 to HL and ALCL cell lines. Binding of bsG1-huCD3xCD30-MDX060 to (A) HDLM-2 (HL), (B) L-428 (HL), (C) DEL (ALCL), or (D) KI-JK (ALCL) cells was evaluated by flow cytometry. Bispecific antibodies bsG1-huCD3xb12 and bsG1-

b12xCD30-MDX060 and monospecific antibodies IgG1-CD30-MDX060, IgG1-huCD3 and IgG1-12 were included as controls. All antibodies contained the FEAL and/or FERR Fc silencing and DuoBody® technology mutations in their Fc domain as indicated. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 3: Induction of cytotoxicity *in vitro* by CD3xCD30 bispecific antibodies in SU-DHL-1 or HDML-2 cells. CD3xCD30 bispecific antibodies were tested in an in vitro cytotoxicity assay using the CD30-positive tumor cell lines SU-DHL-1 cells (left panels) or HDLM-2 cells (right panels) as target cells and T cells (CD3-positive ADCC effector cells type IV cells; Clean Cells, Montaigu, France) as effector cells. The following antibodies were tested: (A) bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-b12-FEALxCD30-MDX060-FEAR, and IgG1-CD30-MDX060-FEAR, (B) bsG1-huCD3-FEALxCD30-hAC10-FEAR and IgG1-CD30-hAC10-FEAR, (C) bsG1-huCD3-FEALxCD30-HRS-3-FEAR and IgG1-CD30-HRS-3-FEAR, (D) BsIgG1-huCD3-FEALxCD30-HeFi-I-FEAR and IgG1-CD30-HeFi-I-FEAR, (E) bsIgG1-huCD3-FEALxCD30-T405-FEAR and IgG1-CD30-T405-FEAR, (F) bsIgG1-huCD3-FEALxCD30-T105-FEAR and IgG1-CD30-T105-FEAR, (G) bsIgG1-huCD3-FEALxCD30-T408-FEAR and IgG1-CD30-T408-FEAR, or (H) bsIgG1-huCD3-FEALxCD30-T215-FEAR and IgG1-CD30-HRS-3-FEAR. The antibody bsG1-huCD3-FEALxb12-FEAR was included as control in all experiments. Data shown are percentages viable cells; data for each graph were obtained from one representative experiment.

Figure 4: Induction of T-cell mediated cytotoxicity and T-cell proliferation *in vitro* by CD3xCD30 bispecific antibodies in several ALCL and HL cell lines. (A-C) CD3xCD30 bispecific antibodies were tested in an in vitro cytotoxicity assay using different ALCL and HL cell lines as target cells and purified T cells (A, B) or ADCC effector cells type IV cells (C) or as effector cells. CD3xCD30 bispecific antibodies contained the huCD3-FEAL Fab arm or the huCD3-H101G-FEAL variant, which has lower affinity for CD3, and the CD30-specific MDX060-FEAR Fab arm. IgG1-huCD3 and IgG1-b12 (A, B) or bsG1-b12-FEALxCD30-MDX060-FEAR and IgG1-CD30-MDX060-FEAR (C) were included as controls. Data shown are percentages viable cells; data for each graph were obtained from one representative experiment. (D) The number of CFSE-positive cells was evaluated as a measure of absolute T-cell counts in the cytotoxicity assays using HDLM-2 cells (left panel) or NCEB-1 cells (right panel) as target cells.

Figure 5: Binding of CD3xCD30 bispecific antibodies to full length human and cynomolgus monkey CD30 transfected into Expi293F cells. (A-C) Binding of monovalent and bivalent CD30 antibodies to wild-type Expi293F cells (A) or Expi293F cells transiently transfected with full length human CD30 (B) or cynomolgus monkey CD30 (C). Cells were incubated with increasing concentrations of the following antibodies: IgG1-CD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR,

bsG1-b12-FEALxCD30-MDX060-FEAR, and bsG1-huCD3-FEALxb12-FEAR. Data are presented as mean fluorescence intensity (MFI) values as determined by flow cytometry of two technical replicates. (D) Binding of antibodies IgG1-CD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-MDX060-FEAR, and bsG1-huCD3-FEALxb12-FEAR to human T cells or cynomolgus monkey T cells. Data are presented as mean fluorescence intensity (MFI) values as determined by flow cytometry of one representative experiment.

Figure 6: Binding of CD3xCD30 bispecific antibodies to full length human and rhesus monkey CD30 transfected into Expi293F cells. Binding of monovalent and bivalent CD30 antibodies to Expi293F cells transiently transfected with full length human CD30 (left panels) or rhesus monkey CD30 (right panels) was evaluated by flow cytometry. The following antibodies were evaluated: (A) bsG1-huCD3-FEALxCD30-hAC10-FEAR and IgG1-CD30-hAC10-FEAR, (C) bsG1-huCD3-FEALxCD30-HRS-3-FEAR and IgG1-CD30-HRS-3-FEAR, (D) bsIgG1-huCD3-FEALxCD30-HeFi-I-FEAR and IgG1-CD30-HeFi-I-FEAR, (E) bsIgG1-huCD3-FEALxCD30-T405-FEAR and IgG1-CD30-T405-FEAR, (F) bsIgG1-huCD3-FEALxCD30-T105-FEAR and IgG1-CD30-T105-FEAR, (G) bsIgG1-huCD3-FEALxCD30-T408-FEAR and IgG1-CD30-T408-FEAR, or (H) bsIgG1-huCD3-FEALxCD30-T215-FEAR and IgG1-CD30-HRS-3-FEAR. The antibody bsG1-huCD3-FEALxb12-FEAR was included as a negative control in all experiments. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 7: Thermostability of antibodies with different non-activating mutations as determined by differential scanning fluorimetry (DSF). Conformational protein stability at increasing temperatures was evaluated in duplicate by DSF. Melting curves of the following antibodies are shown: (A) IgG1-CD30-MDX060-FEAR at pH 7.4, (B) IgG1-CD30-MDX060-FERR at pH 7.4, (C) IgG1-huCD3-FEAL at pH 7.4, and (D) BsG1-huCD3-FEALxCD30-MDX060-FERR at pH 7.4.

Figure 8: Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to HL cell lines.
Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to HL cell lines L-540 (A), KM-H2 (B), and L-1236 (C) was evaluated by flow cytometry. Bispecific antibodies bsG1-huCD3-FEALxb12-FERR and bsG1-b12-FEALxCD30-MDX060-FERR and monospecific antibodies IgG1-CD30-MDX060-FERR, IgG1-huCD3-FEAL and IgG1-12-FEAL were included as controls. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 9: Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to ALCL cell lines.
Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to ALCL cell lines SUP-M2 (A), DL-40 (B), KARPAS-299 (C), L-82 (D), and SR-786 (E) was evaluated by flow cytometry. Bispecific antibodies bsG1-huCD3-FEALxb12-FERR and bsG1-b12-FEALxCD30-MDX060-FERR and

monospecific antibodies IgG1-CD30-MDX060-FERR, IgG1-huCD3-FEAL and IgG1-12-FEAL were included as controls. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 10: Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to NHL cell lines. Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to (A) SUP-T1 (TLL), (B) JVM-2 (MCL), (C) HH (CTCL), and (D) NCEB-1 (MCL) cell lines was evaluated by flow cytometry. Bispecific antibodies bsG1-huCD3-FEALxb12-FERR and bsG1-b12-FEALxCD30-MDX060-FERR and monospecific antibodies IgG1-CD30-MDX060-FERR, IgG1-huCD3-FEAL and IgG1-12-FEAL were included as controls. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 11: Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to full length human and rhesus monkey CD30 transfected into HEK293 cells. Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to HEK293 cells transiently transfected with full length human CD30 (A) or rhesus monkey CD30 (B) was evaluated by flow cytometry. bsG1-huCD3-FEALxb12-FERR was included as a negative control. Data shown are mean fluorescence intensity (MFI) values as determined by flow cytometry, for one representative experiment.

Figure 12: Simultaneous binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to T cells and tumor cells. Simultaneous binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to fluorescently labelled tumor cells and naive T cells was studied by flow cytometry. (A) Double-positive events are shown as percentage of total viable cells in the presence of 6×10^{-5} to 10 µg/mL bsG1huCD3FEALxCD30-MDX060-FERR or control antibodies bsG1-huCD3-FEALxb12-FEAR, bsG1b12FEALxCD30MDX060-FERR, or IgG1-b12-FEAL. The percentage of double-positive events in samples incubated without antibody are indicated with the dotted line. (B) Example of the gating strategy of double positive cells in samples incubated with 0.12 µg/mL bsG1huCD3FEALxCD30-MDX060-FERR.

Figure 13: Induction of T-cell mediated cytotoxicity and T-cell activation *in vitro* by CD3xCD30 bispecific antibodies.

A panel of CD3xCD30 bispecific antibodies were tested in an *in vitro* cytotoxicity assay using the CD30-positive tumor cell line Karpas-299 as target cells and T cells purified from healthy human donor buffy coats as effector cells. In these assay CD25 expression was evaluated in $CD4^+$ and $CD8^+$ cells as a measure for T-cell activation. The following antibodies were tested: bsG1-huCD3-FEALxCD30-MDX060-FERR, bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-hAC10-FEAR, bsG1-huCD3-FEALxCD30-HRS-3-FEAR, BsIgG1-huCD3-FEALxCD30-HeFi-I-FEAR, bsIgG1-huCD3-FEALxCD30-T105-FEAR, bsIgG1-huCD3-FEALxCD30-T405-FEAR, bsIgG1-huCD3-FEALxCD30-T408-FEAR, and bsIgG1-huCD3-FEALxCD30-T215-FEAR. (A) IC_{50} values for cytotoxicity of Karpas-299 cells by CD3xCD30 antibodies. (B) Percentage of maximum

cytotoxicity of Karpas-299 cells by test antibodies. (C-F) EC_{50} values for induction of CD25 (C-D) or PD-1 (E-F) expression in $CD4^+$ (C, E) or $CD8^+$ (D, F) T cells by CD3xCD30 antibodies. Data were obtained from two independent experiments that were performed with T cells obtained from 6 different donors. Statistical values represent the result of a Wilcoxon matched-pairs singed rank test between the indicated clone and bsG1-huCD3-FEALxCD30-MDX060-FERR. NS: Nonsignificant, *: $p < 0.05$.

Figure 14: T-cell mediated cytotoxicity of cell lines *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR. Dose-dependent T-cell mediated cytotoxicity by bsG1-huCD3-FEALxCD30-MDX060-FERR was tested *in vitro* using L-428 (A), KM-H2 (B), SUP-M2 (C), or KI-JK (D) tumor cell lines as target cells and purified T cells as effector cells. IgG1-huCD3-FEAL, bsG1-huCD3-FEALxb12-FERR, IgG1-CD30-MDX060-FERR, bsG1-b12-FEALxCD30-MDX060-FERR, and IgG1-b12-FEAL were included as controls. Data shown are percentages viable cells, data for each graph were obtained for one representative experiment.

Figure 15: T-cell proliferation *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 and KI-JK cell lines. T-cell proliferation was evaluated in T-cell mediated cytotoxicity assays using L-428 (A, B) or KI-JK (C, D) as target cells. $CD4^+$ (A, C) or $CD8^+$ (B, D) T cells with diluted Celltrace Violet staining were gated and the expansion index, as a measure for T-cell proliferation, was calculated using the proliferation modeling tool from FlowJo.

Figure 16: Expression of T-cell activation marker CD69 *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 and KI-JK cell lines. T-cell activation was evaluated in T-cell mediated cytotoxicity assays using L-428 (A, B) or KI-JK (C, D) as target cells. The expression of the T-cell activation marker CD69 was evaluated in $CD4^+$ (A, C) or $CD8^+$ (B, D) T cells.

Figure 17: Expression of T-cell activation marker CD25 *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 and KI-JK cell lines. T-cell activation was evaluated in T-cell mediated cytotoxicity assays using L-428 (A, B) or KI-JK (C, D) as target cells. The expression of the T-cell activation marker CD25 was evaluated in $CD4^+$ (A, C) or $CD8^+$ (B, D) T cells.

Figure 18: Expression of T-cell activation marker PD-1 *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 and KI-JK cell lines. T-cell activation was evaluated in T-cell mediated cytotoxicity assays using L-428 (A, B) or KI-JK (C, D) as target cells. The expression of the T-cell activation marker PD-1 was evaluated in $CD4^+$ (A, C) or $CD8^+$ (B, D) T cells.

Figure 19: Cytokine and Granzyme B production *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR. The concentration of 14 different cytokines (CD40, IFNγ, IL-10, IL-12, IL-13, IL-1b, IL-2, IL-4, IL-6, IL-8, IP-10, MCP-1, PDL-1, TNFα) and Granzyme B was evaluated in

supernatants that were collected during *in vitro* T cell-mediated cytotoxicity experiments using L-428 as target cells. Cytokine and Granzyme B concentrations are shown for samples treated with different concentrations of bsG1-huCD3-FEALxCD30-MDX060-FERR or control antibody IgG1-b12-FEAL.

Figure 20: T-cell mediated cytotoxicity and T-cell proliferation *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR at varying effector to target ratios. (A) Dose-dependent T-cell mediated cytotoxicity by bsG1-huCD3-FEALxCD30-MDX060-FERR was tested *in vitro* using L-428 tumor cells as target cells and purified T cells as effector cells at E:T ratios of 1:1, 2:1, 4:1 or 8:1. bsG1-huCD3-FEALxb12-FERR was included as control antibody. Data shown are percentages viable cells, obtained from one representative experiment. (B, C) The percentage of $CD4^+$ (B) or $CD8^+$ (C) T cells with diluted Celltrace Violet staining is shown as a measure of proliferating T cells.

Figure 21: Kinetics of T-cell mediated cytotoxicity and T-cell proliferation *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR. (A) The kinetics of T-cell mediated cytotoxicity by bsG1-huCD3-FEALxCD30-MDX060-FERR was tested *in vitro* using L-428 tumor cells as target cells and purified T cells as effector cells at an E:T ratio of 4:1. Cytotoxicity was evaluated after 24 h, 48 h, and 72 h. bsG1-huCD3-FEALxb12-FERR was included as control antibody. Data shown are percentages viable cells, obtained from one representative experiment. (B, C) $CD4^+$ (B) or $CD8^+$ (C) T cells with diluted Celltrace Violet staining were gated and the expansion index, as a measure for T-cell proliferation in the cytotoxicity assays, was calculated using the proliferation modeling tool from FlowJo.

Figure 22: T-cell mediated cytotoxicity *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR and correlation with CD30 expression level. The correlation between maximum T-cell mediated kill (A) or IC50 concentrations of T-cell mediated cytotoxicity (B) by bsG1-huCD3-FEALxCD30-MDX060-FERR versus CD30 expression levels was evaluated in eight tumor cell lines. A Spearman rank correlation test (GraphPad Prism software) was used to evaluate the statistical significance of the degree of correlation between T-cell mediated cytotoxicity (maximum kill or IC_{50}) and CD30 expression.

Figure 23: Fratricide of activated T cells by bsG1-huCD3-FEALxCD30-MDX060-FERR. Isolated healthy donor T cells were stimulated with 1 µg/mL anti-CD3 (OKT-3), 1 µg/mL anti-CD28 and 0.025 µg/mL IL-15 for 4 days. Upon confirmation of activation (CD25 upregulation), T cells were incubated with increasing concentrations of bsG1-huCD3-FEALxCD30-MDX060-FERR, bsIgG1-ctrlxCD30-MDX-060-FERR, bsIgG1-CD3xb12, or IgG1-b12-FEAL for 48 hours. BsG1-huCD3-FEALxCD30-MDX060-FERR-induced T-cell fratricide of activated-$CD30^+$ T-cells was measured as the percentage of viable T cells in each condition relative to the number of viable T cells in the condition without adding any antibodies. (A-B) The percentage of $CD25^+$ (A) or $CD30^+$

(B) cells in $CD4^+$ or $CD8^+$ T-cells, 72- and 96 hours after stimulation with anti-CD3, anti-CD28 and IL-15, was determined by flow cytometry. (C) T-cell fratricide was shown as the percentage T-cell survival relative to the condition without adding any antibodies. Data are shown for one representative T-cell donor.

Figure 24: Soluble CD30 in the supernatant of $CD30^+$ cell cultures and its interference with the anti-tumor activity of BsG1-huCD3-FEALxCD30-MDX060-FERR. (A) The soluble CD30 (sCD30) concentration in the supernatant of different hematologic tumor cell lines, as measured by ELISA, is shown. (B) The correlation between sCD30 concentrations and the number of CD30 molecules on the cell surface, as determined using quantitative flow cytometry (human IgG calibrator kit-Biocytex), in different hematologic tumor cell lines, was evaluated by Spearman rank correlation test (GraphPad Prism software). (C) BsG1-huCD3-FEALxCD30-MDX060-FERR was tested in an *in vitro* cytotoxicity assay using the CD30-positive ALCL tumor cell line DEL as target cells and healthy donor isolated T cells as effector cells. The following control antibodies were included: BsG1-b12-FEALxCD30-MDX060-FERR, IgG1-CD30-MDX060-FERR, BsG1-huCD3-FEALxb12-FERR, IgG1-huCD30-FEAL and IgG1-b12-FEAL. Data shown are percentages viable cells, obtained from one representative experiment.

Figure 25. Induction of T-cell mediated cytotoxicity and T-cell proliferation *ex vivo* by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 tumor cells. (A) bsG1-huCD3-FEALxCD30-MDX060-FERR was tested in an ex vivo cytotoxicity assay using L-428 tumor cells as target cells and primary patient-derived T cells as effector cells. Hodgkin lymphoma (HL), acute myeloid leukemia (AML) and peripheral T-cell lymphoma (PTCL) patient-derived peripheral blood mononuclear cells (PBMCs) were used as a source of T cells to evaluate CD3-dependent tumor cell kill. IgG1-b12-FEAL was included as control. Data shown are percentage viable target cells. (B-D) T-cell activation was evaluated by upregulation of CD69 (B), CD25 (C) and PD-1 (D) markers on $CD4^+/CD8^+$ T cells within the PBMC subset, indicated as percentage positive cells.

Figure 26. Plasma concentrations of BsG1-huCD3-FEALxCD30-MDX060-FERR following intravenous injection in SCID mice. SCID mice were injected with a single IV dose of 10 µg (0.5 mg/kg) or 100 µg (5 mg/kg) of BsG1-huCD3-FEALxCD30-MDX060-FERR. (A) Total human IgG was determined by ELISA and average human IgG1 concentrations were plotted over time. (B) The mean clearance rate was plotted for 0.5 or 5 mg/kg dose levels. The dotted line indicates the estimated clearance rate based on standard distribution volume of a non-binding regular human IgG1 in mice.

Figure 27: C1q binding to membrane-bound bsG1-huCD3-FEALxCD30-MDX060-FERR. Binding of C1q to bsG1-huCD3-FEALxCD30-MDX060-FERR-opsonized, activated human $CD8^+$ T cells, or $CD30^+$ NCEB-1 cells was evaluated by flow cytometry using FITC labeled rabbit anti-C1q antibody. (A) Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR, IgG1-huCD3-FEAL or positive

control antibody IgG1-CD52-E430G to human CD8$^+$ T cells stimulated with anti-CD3/CD28 beads in the presence of normal human serum as a source of C1q. Data shown are geomean fluorescence intensities (gMFI) ± SD from replicate wells from one representative experiment. (B) Binding of bsG1-huCD3-FEALxCD30-MDX060-FERR, IgG1-CD30-MDX060-FERR or positive control antibody IgG1-7D8-E430G to CD30$^+$ NCEB-1 cells, in the presence of normal human serum as a source of C1q. Data shown are gMFI from one representative experiment.

DETAILED DESCRIPTION OF THE INVENTION

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 may 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 human 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 bi-, tri-, or multispecific antibodies and/or antibodies having further modifications, e.g., antibody-drug conjugates and/or antibodies with modifications in the IgG Fc domain. An antibody as defined according to the invention can possess any isotype or not have an isotype (e.g., an scFv antibody), 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, 544546 (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, 423426 (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, either as the final product or as an intermediate to generate e.g., a bispecific antibody through controlled Fab-arm exchange (cFEA), 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 that use cellular extracts supporting *in vitro* transcription and/or translation of nucleic acid sequences encoding the antibody.

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 of an IgG typically is comprised of three domains, CH1, CH2, and CH3. The term "immunoglobulin" as used herein is intended to refer to a class of structurally related glycoproteins typically 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 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 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 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, 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 terms "first" and "second" when used herein in the context of an antibody or domain or region thereof are merely intended for ease of reference and are not intended to indicate a particular relative location or the like.

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, protein, polysaccharide or combination thereof. 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 "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, such as $1E^{-9}$ M or less, such as $1E^{-10}$ M or less or such as $1E^{-11}$ 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 "CD30" as used herein refers to the human Cluster of Differentiation 30 protein, also known as TNFRSF8 (Tumor Necrosis Factor Receptor Superfamily member 8). CD30 is found in various species, and thus, the term "CD30" may not be limited to human CD30, unless contradicted by context. The sequence of human CD30 is set forth in SEQ ID NO:39.

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:42; 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 or other agents that disrupt the three-dimensional structure of a protein or multimer thereof. 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.

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 trans-chromosomal 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 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. Thus, for example, a full-length IgG1 antibody contains VH, CH1, CH2, CH3, hinge, VL and CL domains. 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 some of the human framework residues with some of the framework residues from the parental antibody (i.e. the non-human antibody) (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, and/or improve manufacturing. Furthermore, CDR and/or framework regions may be modified to improve affinity to the antigen, for example via affinity maturation procedures.

"The term "human antibody" as used herein, refers to antibodies having variable and constant regions derived from human germline immunoglobulin sequences. Human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Human monoclonal antibodies of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology, *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can

be employed, *e.g.*, viral or oncogenic transformation of B-lymphocytes or phage display techniques using libraries of human antibody genes. Human monoclonal antibodies can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system, such as a HCo12 mouse (see e.g. WO 03/059282), or the like.

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 chain polypeptides of the antibody, at least a hinge region, a CH2 region and a CH3 region. The Fc polypeptides are typically glycosylated. 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. Fc regions typically also bind FcRn and protein A.

The phrase "amino acid corresponding to the amino acid at position…", or the like, 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 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.

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 according to the Eu numbering as set forth 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). 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 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 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 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 cytotoxicity (ADCC), (v) Fc-gamma receptor (FcgR)-binding, (vi) antibody-dependent, FcγR-mediated antigen crosslinking, (vii) antibody-dependent cellular phagocytosis (ADCP), (viii) complement-dependent cellular 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 not able or has minimal ability to bind any FcγR, induce Fc-mediated cross-linking of FcγRs, induce FcγR-mediated effector functions such as ADCC and ADCP, induce FcγR-mediated cross-linking of target antigens via two Fc regions of individual antibodies, and/or is not able to bind C1q and induce complement-mediated effector functions such as CDC and CDCC. The inertness of an Fc region of an antibody, may be tested using the antibody in a monospecific or bispecific format.

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 multispecific, such as a bispecific, antibody, "monovalent antibody binding" refers to the binding of the multispecific 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), a monospecific, bivalent antibody (e.g. an antibody with two identical antigen-binding regions) or a monospecific, multivalent antibody (e.g. an antibody with more than two identical antigen-binding regions).

The term "multispecific antibody" refers to an antibody having two or more antigen-

binding domains that bind two or more different epitopes. 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 and multispecific antibodies have specificity for two or more, respectively, 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. Multispecific and bispecific antibodies may thus be capable of crosslinking multiple antigens, e.g. two different cells.

The term "bivalent antibody" refers to an antibody that has two antigen-binding regions, where said antigen-binding regions may be identical and bind to the same epitopes or they may be non-identical and bind to different epitopes which may be on the same or on 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 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:

TABLE 1

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

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 2

Alternative Physical and Functional Classifications of Amino Acid Residues

Class	Amino acid
Hydroxyl group containing	S and T

residues	
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 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, a conservative substitution may be substitution of leucine with isoleucine as they are both aliphatic, branched hydrophobes. Similarly, an example of a conservative substitution is substitution of aspartic acid with glutamic acid since they are both small, negatively charged residues.

In the context of the present invention, a substitution in an antibody is indicated as: Original amino acid – number of 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 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 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" 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. 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 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 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 (i.e. host cells used for the production of recombinant proteins) 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.

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 (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 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.

TABLE 3 Amino acid sequences

SEQ ID NO:	Reference	Domain	Sequence
1	IgG1-CD30-MDX060-FERR	VH_CDR1	GGSFSAYY
2	IgG1-CD30-MDX060-FERR	VH_CDR2	INHGGGT
3	IgG1-CD30-MDX060-FERR	VH_CDR3	ASLTAY
4	IgG1-CD30-MDX060-FERR	VL_CDR1	QGISSW
5	IgG1-CD30-MDX060-FERR	VL_CDR2	AAS
6	IgG1-CD30-MDX060-FERR	VL_CDR3	QQYDSYPIT
7	IgG1-huCD3-FEAL	VH_CDR1	GFTFNTYA
8	IgG1-huCD3-FEAL	VH_CDR2	IRSKYNNYAT
9	IgG1-huCD3-FEAL	VH_CDR3	VRXGNFGNSYVSWFAY, wherein X is H or G
10	IgG1-huCD3-FEAL	VL_CDR1	TGAVTTSNY
11	IgG1-huCD3-FEAL	VL_CDR2	GTN
12	IgG1-huCD3-FEAL	VL_CDR3	ALWYSNLWV

13	IgG1-CD30-MDX060-FERR	VH	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSAYYWSWIRQPPGKGLEWIGDINHGGGTNYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCASLTAYWGQGSLVTVSS
14	IgG1-CD30-MDX060-FERR	VL	DIQMTQSPTSLSASVGDRVTITCRASQGISSWLTWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYDSYPITFGQGTRLEIK
15	IgG1-huCD3-FEAL	VH	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKSSLYLQMNNLKTEDTAMYYCVRXGNFGNSYVSWFAYWGQGTLVTVSS, wherein X is H or G
16	IgG1-huCD3-FEAL	VL	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNKRAPGVPARFSGSLIGDKAALTITGAQADDESIYFCALWYSNLWVFGGGTKLTVL
17	IgG1-CD30-MDX060-FERR	Full heavy chain	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSAYYWSWIRQPPGKGLEWIGDINHGGGTNYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCASLTAYWGQGSLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFERGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
18	IgG1-CD30-MDX060-FERR	Full light chain	DIQMTQSPTSLSASVGDRVTITCRASQGISSWLTWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYDSYPITFGQGTRLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

19	IgG1-huCD3-FEAL	Full heavy chain	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKSSLYLQMNNLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
20	IgG1-huCD3-FEAL	Full light chain	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNKRAPGVPARFSGSLIGDKAALTITGAQADDESIYFCALWYSNLWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
21	IgG1-CD30-hAC10-FEAR	Full heavy chain	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYYITWVRQAPGQGLEWMGWIYPGSGNTKYNEKFKGRVTMTRDTSISTAYMELSRLRSDDTAVYYCANYGNYWFAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

22	IgG1-CD30-hAC10-FEAR	Full light chain	DIVMTQSPDSLAVSLGERATINCKASQSVDFDGDSYMNWYQQKPGQPPKLLIYAASNLESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQSNEDPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
23	IgG1-CD30-HRS-3-FEAR	Full heavy chain	QVQLQQSGAELARPGASVKMSCKASGYTFTTYTIHWVRQRPGHDLEWIGYINPSSGYSDYNQNFKGKTTLTADKSSNTAYMQLNSLTSEDSAVYYCARRADYGNYEYTWFAYWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
24	IgG1-CD30-HRS-3-FEAR	Full light chain	DIVMTQSPKFMSTSVGDRVTVTCKASQNVGTNVAWFQQKPGQSPKVLIYSASYRYSGVPDRFTGSGSGTDFTLTISNVQSEDLAEYFCQQYHTYPLTFGGGTKLEINRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

25	IgG1-CD30-HeFi-I-FEAR	Full heavy chain	EVKLVESGGGLVQPGGSLRLSCATSGFTFSDYYMNWVRQPPGKALEWLGFIRNKANGYTTEFSASVMGRFTISRDDSQSILYLQMNTLRAEDSATYYCARDPPYGNPHYYAMDYWGQGTSVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
26	IgG1-CD30-HeFi-I-FEAR	Full light chain	DIVLTQSPASLAVSLGQRATISCRASKSVSASGYNYMHWYQQKAGQPPKLLIHLASNLESGVPARFSGSGSGTDFTLNIHPVEEEDASTYYCQHSGELPFTFGSGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
27	IgG1-CD30-T405-FEAR	Full heavy chain	QVQLQQIGAELVRPGASVKLSCKASGYTFNNYWINWVKQRPGQGLEWIGNIYPSDSRSNYNQKFKDKATLTVDKPSSTAYMQLSSPTSEDSAVYYCTLGSYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

28	IgG1-CD30-T405-FEAR	Full light chain	DVVMTQTPLTLSVTIGQPASISCKSSQSLSDSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGAHFPRTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
29	IgG1-CD30-T105-FEAR	Full heavy chain	QVTLKESGPGILQPSQTLSLTCSFSGFSLSTSGMGVSWIRQPSGKDLEWLAHIYWDDDKRYNPSLKSRLTISKDTSSNQVFLKITSVDTADTATYYCARRADGLYFYLDVWGAGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
30	IgG1-CD30-T105-FEAR	Full light chain	DIVMTQSQKFMSTSVGDRVSVTCKASQNVNTNVAWYQQKPGQSPEALIYSASYRYSGVPDRFTGSGSGTDFTLTISNVQSEDLAEYFCQQYNSYPLTFGSGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

31	IgG1-CD30-T408-FEAR	Full heavy chain	QVQLQQPGAELVRPGASVKLSCKASGYTFTSYWINWVKQRPGQGLEWIGNIYPSDSYSNYNQKFKDKATLTVDKSSSTAYMQLSSPTSEDSAVYYCTLGSYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
32	IgG1-CD30-T408-FEAR	Full light chain	DVVMTQTPLTLSVTIGQPASISCKSSQSLSDSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGAHFPRTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
33	IgG1-CD30-T215-FEAR	Full heavy chain	QVTLKESGPGILQPSQTLSLTCSFSGFSLSVSGMGVSWIRQPSGKGLEWLAHIHWDDDRRSNPSLRSRLTISKDTSNNQVFLKITSVDTADTATYYCARRPDLYGNYFFFDFWGQGTTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

34	IgG1-CD30-T215-FEAR	Full light chain	DIVMTQSQKFMSTSVGDRVSVTCKASQNVFTNVAWYQQKLGQSPKPLIYSASYRYSGVPDRFTGSGSGTDFTLTISDVQSEDLAEYFCQQYNSYPVTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
35	IgG1-huCD3H101G-FEAL	Full heavy chain	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKSSLYLQMNNLKTEDTAMYYCVRGGNFGNSYVSWFAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
36	IgG1-b12-FEAL	Full heavy chain	QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWVRQAPGQRFEWMGWINPYNGNKEFSAKFQDRVTFTADTSANTAYMELRSLRSADTAVYYCARVGPYSWDDSPQDNYYMDVWGKGTTVIVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

37	IgG1-b12-FEAL	Full light chain	EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQH KPGQAPRLVIHGVSNRASGISDRFSGSGSGTDFTLTITR VEPEDFALYYCQVYGASSYTFGQGTKLERKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQ SGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYAC EVTHQGLSSPVTKSFNRGEC
38	IgG1-b12-FERR	Full heavy chain	QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWVR QAPGQRFEWMGWINPYNGNKEFSAKFQDRVTFTADTSA NTAYMELRSLRSADTAVYYCARVGPYSWDDSPQDNYYM DVWGKGTTVIVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCD KTHTCPPCPAPEFERGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG
39	human CD30		FPQDRPFEDTCHGNPSHYYDKAVRRCCYRCPMGLFPTQ QCPQRPTDCRKQCEPDYYLDEADRCTACVTCSRDDLVE KTPCAWNSSRVCECRPGMFCSTSAVNSCARCFFHSVCP AGMIVKFPGTAQKNTVCEPASPGVSPACASPENCKEPSS GTIPQAKPTPVSPATSSASTMPVRGGTRLAQEAASKLTR APDSPSSVGRPSSDPGLSPTQPCPEGSGDCRKQCEPDY YLDEAGRCTACVSCSRDDLVEKTPCAWNSSRTCECRPG MICATSATNSCARCVPYPICAAETVTKPQDMAEKDTTFE APPLGTQPDCNPTPENGEAPASTSPTQSLLVDSQASKTL PIPTSAPVALSSTGKPVLDAGPVLFWVILVLVVVVGSSAF LLCHRRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRS STQLRSGASVTEPVAEERGLMSQPLMETCHSVGAAYLES LPLQDASPAGGPSSPRDLPEPRVSTEHTNNKIEKIYIMKA DTVIVGTVKAELPEGRGLAGPAEPELEEELEADHTPHYPE QETEPPLGSCSDVMLSVEEEGKEDPLPTAASGK

40	Cynomolgous monkey CD30		FPQDRPFEDTCRGNPGHYYDKAVRRCCYRCPTGTAQKN TVCEPASPGVSPACASPENCKEPSSGTIPQAKPTPVSPAT SNASTMPLRGGTRLAQEAASKLTRAPGSPSSVGRPSSDP GLSPTQPCPQGSGDCRKQCEPDYYLDEAGRCTACVSCS RDDLVEKTPCAWNSSRICECRPGMICATSATNSCARCVP YPICAAETGTKPQDMAEKDTTFEAPPVGTQPDCSPTPEN GEAPASTSPTLSSLVDSQASKTLPIPTSAPIALSSTGKPVL DAGPVLFWVILVLAVVVGSSTFLLCHRRACRKRIRQKLHL CYPVQTSRPKLELVDSRPRRSSTQFESGTFVTKPSAEELG LMSLPPKETCRNVGAACPESLPLWDASPAGGPSSPRDLP EPRVSTEHTNNRIEKIYIMKADTVIVGTVKAELPEGQGLA GPAEPELEEELEADHAPHYPEQETEPPLGSCSDVMLSVEE EGKEDPLPTAASGK
41	Rhesus monkey CD30		FPQDRPFEDTCRGNPGHYYDKAVRRCCYRCPMGLFPTQ QCPQRPADCRKQCEPDYYLDEAGRCTACVSCSRDDLVE KMPCAWNSSRVCECQPGMFCAVSVVNSCARCFFHSVCP AGMIVKFPGTAQKNTVCEPASPGVSPACASPENCKEPSS GTIPQAKPTPVSPATSNASTMPLRGGTRLAQEAASKLTR APGSPSSVGRPSSDPGLSPTQPCPQGSGDCRKQCEPDY YLDEAGRCTACVSCSRDDLVEKTPCAWNSSRICECRPG MICATSATNSCARCVPYPICAAETGTKPQDMAEKDTTFE APPVGTQPDCSPTPENGEAPASTSPTLSSLVDSQASKTLP IPTSAPIALSSTGKPVLDAGPVLFWVILVLAVVVGSSTFLL CHRRACRKRIRQKLHLCYPVQTSRPKLELVDSRPRRSST QFESGTFVTKPSAEELGLMSLPPKETCRNVGAACPESLPL WDASPAGGPSSPRDLPEPRVSTEHTNNRIEKIYIMKADT VIVGTVKAELPEGQGLAGPAEPELEEELEADHAPHYPEQE TEPPLGSCSDVMLSVEEEGKEDPLPTAASGK
42	human CD3 (epsilon)		QDGNEEMGGITQTPYKVSISGTTVILTCPQYPGSEILWQ HNDKNIGGDEDDKNIGSDEDHLSLKEFSELEQSGYYVCY PRGSKPEDANFYLYLRARVCENCMEMDVMSVATIVIVDI CITGGLLLLVYYWSKNRKAKAKPVTRGAGAGGRQRGQN KERPPPVPNPDYEPIRKGQRDLYSGLNQRRI

43	Cynomolgus monkey CD3		QDGNEEMGSITQTPYQVSISGTTVILTCSQHLGSEAQW QHNGKNKEDSGDRLFLPEFSEMEQSGYYVCYPRGSNPE DASHHLYLKARVCENCMEMDVMAVATIVIVDICITLGLLL LVYYWSKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVP NPDYEPIRKGQQDLYSGLNQRRI
44	IgG1 constant region		ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPG
45	IgG1 constant region	with FER substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**FE R**GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPG
46	IgG1 constant region	with FEA substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**FE** GGPSVFLFPPKPKDTLMISRTPEVTCVVV**A**VSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPG

47	IgG1 constant region	with F405L substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF**L**LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
48	IgG1 constant region	with K409R substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS**R**LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
49	IgG1 constant region	with FER substitution and with F405L substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**FER**GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF**L**LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

50	IgG1 constant region	with FER substitution and with K409R substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**FER**GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS**R**LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
51	IgG1 constant region	with FEA substitution and with F405L substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**FE**GGPSVFLFPPKPKDTLMISRTPEVTCVVV**A**VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF**L**LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
52	IgG1 constant region	with FEA substitution and with K409R substitution (bold)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**FE**GGPSVFLFPPKPKDTLMISRTPEVTCVVV**A**VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS**R**LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
53	constant region	constant region of human kappa light chain	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

54	constant region	constant region of human lambda light chain	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTV AWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQ WKSHRSYSCQVTHEGSTVEKTVAPTECS
55	IgG1-CD30-MDX060-FEAR	Full heavy chain	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSAYYWSWIR QPPGKGLEWIGDINHGGGTNYNPSLKSRVTISVDTSKN QFSLKLNSVTAADTAVYYCASLTAYWGQGSLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEGGP SVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSDGSFFLYSRLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPG

Further aspects and embodiments of the invention

As described above, in a first aspect, the invention relates to a multispecific antibody comprising:
(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and
(ii) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively.

In one embodiment, the X in SEQ ID NO:9 is an H. In another embodiment, the X in SEQ ID NO:9 is a G.

In one embodiment, the first heavy chain variable region is human or humanized. In another embodiment, the first light chain variable region is human or humanized. In one embodiment, the second heavy chain variable region is human or humanized. In another embodiment, the second light chain variable region is human or humanized. In another embodiment, the first heavy chain variable region and the first light chain variable region are human. In another embodiment, the first heavy chain variable region and the first light chain

variable region are humanized. In another embodiment, the second heavy chain variable region and the second light chain variable region are human. In another embodiment, the second heavy chain variable region and the second light chain variable region are humanized. In another embodiment, the first heavy chain variable region and the first light chain variable region are human and the second heavy chain variable region and the second light chain variable region are human. In another embodiment, the first heavy chain variable region and the first light chain variable region are human and the second heavy chain variable region and the second light chain variable region are humanized.

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. This structure is preferably also found in the antibodies according to the present invention. In one embodiment, one, two, three or all of said four framework sequences are human framework sequences.

As described above, the multispecific antibody according to the invention comprises an antigen-binding region capable of binding to human CD30, the sequence of which is set forth in SEQ ID NO:39. In particular, the antibody according to the invention is an antibody wherein said antigen-binding region capable of binding to human CD30 is capable of binding to the extracellular domain of human CD30, such as a CD30 molecule expressed on a cell, more preferably a tumor cell.

As described, the antibody comprises a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively.

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 have been annotated according to IMGT (see Lefranc, M.-P., The Immunologist, 7, 132-136 (1999); Lefranc, Developmental and Comparative Immunology, 27(1), 55–77 (2003)).

In one embodiment, the antigen-binding region that binds to CD30 comprises:

- a (first) heavy chain variable region (VH) comprising the sequence of SEQ ID NO:13, 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:13; and,
- a (first) light chain variable region (VL) comprising the sequence of SEQ ID NO:14 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:14.

In a further embodiment, the antibody according to the invention comprises a heavy chain variable (VH) region of the antigen-binding region that binds to CD30 as defined herein, wherein the sequences comprises, in total, at the most 1, 2, 3, 4 or 5 amino acid substitutions, when compared with SEQ ID NO:13.

In a further embodiment, the antibody according to the invention comprises a light chain variable (VL) region of the antigen-binding region that binds to CD30 as defined herein, wherein the sequences comprises, in total, at the most 1, 2, 3, 4 or 5 amino acid substitutions, when compared with SEQ ID NO:14.

In a further embodiment, the first heavy chain variable region comprises the sequence set forth in SEQ ID NO:13 and the first light chain variable region comprises the sequence set forth in SEQ ID NO:14.

Such antigen-binding regions that are capable of binding human CD30 have been described i.a. in WO03059282 (Medarex), which is incorporated by reference herein.

The said antibody in accordance with the invention, may bind with an equilibrium dissociation constant KD between the antigen-binding region that binds to human CD30, and human CD30 is within the range of 0.1 to 20 nM, for example within the range of 0.5-5 nM, such as within the range of 1.5-2 nM (monovalent binding). Said binding affinity can be determined by biolayer interferometry.

In one embodiment, the antibody of the invention can also bind cynomolgus CD30 (SEQ ID NO:40).

As described above, the multispecific antibody according to the invention comprises an antigen-binding region capable of binding to human CD3. Furthermore, the invention provides an antibody according to the invention capable of binding human CD3ε (epsilon), such as human CD3ε (epsilon) as specified in SEQ ID NO:22. Such antigen-binding region is capable of binding human CD3ε (epsilon), as presented on a T cell, such as a primary human T cell.

As described, the antibody comprises a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively.

In one embodiment, the antigen-binding region that binds to CD3 comprises:

- a (second) heavy chain variable region (VH) comprising the sequence of SEQ ID NO:15, 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:15; and,
- a (second) light chain variable region (VL) 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.

In a further embodiment, the antibody according to the invention comprises a heavy chain variable (VH) region of the antigen-binding region that binds to CD3 as defined herein, wherein the sequences comprises, in total, at the most 1, 2, 3, 4 or 5 amino acid substitutions, when compared with SEQ ID NO:15.

In a further embodiment, the antibody according to the invention comprises a light chain variable (VL) region of the antigen-binding region that binds to CD3 as defined herein, wherein the sequences comprises, in total, at the most 1, 2, 3, 4 or 5 amino acid substitutions, when compared with SEQ ID NO:16.

In a further embodiment, the second heavy chain variable region comprises the sequence set forth in SEQ ID NO:15 and the second light chain variable region comprises the sequence set forth in SEQ ID NO:16. In one embodiment, the X in SEQ ID NO:15 is an H. In another embodiment, the X in SEQ ID NO:15 is a G.

Such antigen-binding regions that are capable of binding human CD3 have been described i.a. in WO2015/001085 (Genmab), which is incorporated by reference herein. Variants hereof, such as variants comprising a VH CDR3 region as set forth in SEQ ID NO:9 wherein X is G, which has a lower affinity for human CD3 binding than the parental antibody wherein X is H, have been described in Example 2 of WO2017/009442 (Genmab), which is herewith incorporated by reference.

The said antibody in accordance with the invention, may bind with an equilibrium dissociation constant KD between the antigen-binding region that binds to human CD3, and human CD3 is within the range of 5 to 30 nM, such as between 10 and 20 nM (for monovalent binding).

In one embodiment, the antibody of the invention can also bind cynomolgus monkey CD3 (SEQ ID NO:43).

In a further embodiment, the multispecific antibody comprises:

(i) a CD30 binding region comprising a first heavy chain variable region comprising the sequence set forth in SEQ ID NO:13 and a first light chain variable region comprising the sequence set forth in SEQ ID NO:14, and

(ii) a CD3 binding region comprising a second heavy chain variable region comprising the sequence set forth in SEQ ID NO:15 and a second light chain variable region comprising the sequence set forth in SEQ ID NO:16.

Antibody formats

The multispecific antibody of the invention, may have two or more specificities, such as two or three or more specificities. Furthermore, the multispecific antibody may have more than one copy of the antigen-binding region for CD3 and/or CD30. For example, in one embodiment, the

antibody has two antigen-binding regions capable of binding CD3, such as two identical binding regions that bind CD3. For example, in another embodiment, the antibody has two antigen-binding regions that bind CD30, such as two identical binding regions that bind CD30. An additional antigen-binding region may e.g. be present in the form of a scFv covalently linked to the constant region.

In a preferred embodiment, the multispecific antibody of the invention is a bispecific antibody. 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 and by Labrijn et al. 2019 Nat Rev Drug Discov 18(8) 585-608. 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 targets, 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.

Further 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), kappa/lambda body™ molecules (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 et al. 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 100th Annual meeting 2009 (Abstract # 5465); Zymogenetics/BMS, WO2010111625), Dual Affinity Retargeting Technology (Fc-based

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.), 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.

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 (such as described in WO2011131746 (Genmab)).

In one embodiment, the antibody of the present invention is a bispecific DuoBody® molecule (Genmab A/S, WO2011131746).

The multispecific, such as bispecific, antibody of the 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, the 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, or both may be used, e.g. the sequences set forth in SEQ ID N:53 and 54. For example, in one embodiment, the light chain involved in CD30 binding comprises a kappa constant region and the light chain involved in CD3 binding comprises a lambda constant region. 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 region sequences thereof of the selected isotype, may be modified, preferably in the hinge, CH2 and/or CH3 region, to enable the generation of bispecific antibodies and/or introduce inertness.

In one embodiment, the multispecific antibody of the invention comprises an Fc region consisting of a first and second Fc polypeptide.

In one embodiment, the first Fc polypeptide and the first heavy chain variable region are comprised within the same polypeptide chain and wherein the second Fc polypeptide and the second heavy chain variable region are comprised within the same polypeptide chain.

The first and second Fc polypeptide 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. In one embodiment, said Fc region is a human IgG1 Fc region.

In a further embodiment, the multispecific antibody is a full-length antibody as defined herein.

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 that it does not comprise said modifications. 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 e.g. as described in WO2015001085), 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. by flow cytometry 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%, for example in a linear part of the curve, 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).

Examples of amino acid positions that may be modified, e.g. in an IgG1 isotype antibody, include positions L234 and L235. In one embodiment, the first and/or second Fc polypeptide comprise(s) a substitution of an amino acid corresponding to the amino acid at positions L234 and/or L235 in a human IgG1 heavy chain, wherein the substitutions preferably are to F and E, respectively, wherein the amino acid positions are as defined by Eu numbering.

It is understood that in addition to modifications of amino acid positions L234 and L235, further positions may be modified. Thus, in a further embodiment, the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and the first and/or second Fc polypeptide further comprises a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R.

In another embodiment, the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and the first and second Fc polypeptide further comprise a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R.

In another embodiment, the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and the first and/or second Fc polypeptide further comprises a substitution of an amino acid corresponding to the amino acid at position D265 in a human IgG1 heavy chain, wherein the substitution preferably is to A.

In another embodiment, the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and the first and second Fc polypeptide further comprises a substitution of an amino acid corresponding to the amino acid at position D265 in a human IgG1 heavy chain, wherein the substitution preferably is to A.

In another embodiment, one of the first and second Fc polypeptides comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and G236 to F, E and R, respectively, and the other Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235E and D265 to F, E and A, respectively.

In a further embodiment, the first Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and G236 to F, E and R, respectively, and the second Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235E and D265 to F, E and A, respectively, wherein the amino acid positions are as defined by Eu numbering.

For example, constant regions having such Fc region substitutions are provided i.a. in SEQ ID NO. 45, 46, 49, 50, 51 and 52, which can be compared with SEQ ID NO. 44, which does not have such substitution(s). In one embodiment, the antibody of the invention comprises a sequence selected from the group consisting of SEQ ID NO:45, 46, 49, 50, 51 and 52.

In one embodiment, the multispecific or bispecific antibody of the invention comprises an Fc region comprising first and second CH3 regions that 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. 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 antibodies containing only a few, asymmetrical mutations in the CH3 regions.

In particular embodiments, the invention provides an antibody, wherein the amino acid in the position corresponding to K409 in a human IgG1 heavy chain is R in said first Fc polypeptide, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain is L in said second Fc polypeptide, or vice versa.

In a further embodiment, the amino acid in the position corresponding to K409 is R in the first Fc polypeptide and wherein the amino acid in the position corresponding to F405 is L in the second Fc polypeptide.

Hence, in one embodiment, in the first Fc polypeptide, 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 heavy chain has been substituted, and in the second Fc polypeptide 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 heavy chain has been substituted, and wherein said substitutions in the first and second Fc polypeptides are not in the same positions, wherein the amino acid positions are as defined by Eu numbering. For example, constant regions having such Fc region substitutions are provided i.a. in SEQ ID NO. 47, 48, 49, 50, 51 and 52, which can be compared with SEQ ID NO. 44, which does not have such a substitution. In one embodiment, the antibody of the invention comprises a sequence selected from the group consisting of SEQ ID NO: 47, 48, 49, 50, 51 and 52.

Preferably, the amino acid in the position corresponding to F405 is L in the first Fc polypeptide and the amino acid in the position corresponding to K409 is R in the second Fc polypeptide, or vice versa.

Thus, in one embodiment, one of the first and second Fc polypeptides comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235, G236 and F405 to F, E, R and L, respectively, and the other Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235E, D265 and K409 to F, E, A and R, respectively.

In another embodiment, one of the first and second Fc polypeptides comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235, G236 and K409 to F, E, R and R, respectively, and the other Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235E, D265 and F405 to F, E, A and L, respectively.

In a further embodiment, the invention relates to a multispecific antibody comprising:

(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively;

wherein the multispecific antibody is a bispecific antibody and comprises an Fc region consisting of a first and second Fc polypeptide;

wherein the first Fc polypeptide and the first heavy chain variable region are comprised within the same polypeptide chain and wherein the second Fc polypeptide and the second heavy chain variable region are comprised within the same polypeptide chain;

wherein the first Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and G236 to F, E and R, respectively, and the second Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and D265 to F, E and A, respectively, wherein the amino acid positions are as defined by Eu numbering; and

wherein the amino acid in the position corresponding to K409 is R in the first Fc polypeptide and wherein the amino acid in the position corresponding to F405 is L in the second Fc polypeptide.

In a further embodiment, the invention relates to a multispecific antibody comprising:

(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively;

wherein the multispecific antibody is a bispecific antibody and comprises an Fc region consisting of a first and second Fc polypeptide;

wherein the first Fc polypeptide and the first heavy chain variable region are comprised within the same polypeptide chain and wherein the second Fc polypeptide and the second heavy chain variable region are comprised within the same polypeptide chain;

wherein the first Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and D265 to F, E and A, respectively, and the second Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and D265 to F, E and A, respectively, wherein the amino acid positions are as defined by Eu numbering; and

wherein the amino acid in the position corresponding to K409 is R in the first Fc polypeptide and wherein the amino acid in the position corresponding to F405 is L in the second Fc polypeptide.

In one embodiment, the antibody of the invention comprises or consists of the heavy chain sequences set forth in SEQ ID NO:17 and 19 and the light chain sequences set forth in SEQ ID NO:18 and 20.

In a further embodiment, the antibody of the invention comprises or consists of the heavy chain sequences set forth in SEQ ID NO:17 and 19 and the light chain sequences set forth in SEQ ID NO:18 and 20, and said antibody is a bispecific antibody.

In one embodiment, the antibody of the invention comprises or consists of the heavy chain sequences set forth in SEQ ID NO:17 and 35 and the light chain sequences set forth in SEQ ID NO:18 and 20.

In one embodiment, the antibody of the invention comprises or consists of the heavy chain sequences set forth in SEQ ID NO:55 and 19 and the light chain sequences set forth in SEQ ID NO:18 and 20.

In one embodiment, the antibody of the invention comprises or consists of the heavy chain sequences set forth in SEQ ID NO:55 and 35 and the light chain sequences set forth in SEQ ID NO:18 and 20.

In a still further embodiment, the antibody of the invention is bsG1-huCD3-FEALxCD30-MDX060-FEAR or bsG1-huCD3-FEALxCD30-MDX060-FERR. In an even further embodiment, the antibody of the invention is bsG1-huCD3-FEALxCD30-MDX060-FERR.

The constant region sequences listed in SEQ ID NOs: 44-52 and 55 do not include a C-terminal lysine (K). However, in naturally occurring sequences found in humans from which these Fc regions are derived, such a C-terminal lysine may be present as part of the open reading frame. During cell culture production of recombinant antibodies, this terminal lysine may 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 may be omitted from the sequence such that antibodies are produced without the lysine. Omission of the C-terminal lysine from the sequence encoding the antibody may increase the homogeneity of the antibody with respect to the presence of C-terminal lysine. Antibodies 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 C-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 C-terminal lysine such as listed herein. For manufacturing purposes, antibodies can thus be generated without having a C-terminal lysine.

In alternative embodiments, the multispecific antibody according to the invention in not a classical full-length antibody comprising an Fc region. For example, in one embodiment,

(i) the CD30 binding region and/or the CD3 binding region is a Fab,
(ii) the CD30 binding region and/or the CD3 binding region is an scFv,
(iii) the CD30 binding region is Fab and the CD3 binding region is an scFv, or
(iv) the CD30 binding region is an scFv and the CD3 binding region is a Fab.

<u>Binding, cytotoxicity and T-cell activation</u>

Antibodies, such as bispecific antibodies, as described herein that can bind to human CD3 and human CD30 can advantageously target T cells to human CD30 expressing cancer cells, thereby inducing T-cell mediated killing of said cancer cells.

As said, preferably, the antibody in accordance with the invention is inert, and furthermore, the antibody comprises one or more of the following characteristics:

a) is capable of binding to CD30-expressing human tumor cells, such as SU-DHL-1 cells, SUP-M2 cells, DL-40 cells, KARPAS-299 cells, L-82 cells, SR-786 cells, L-540 cells, KM-H2 cells, L-1236 cells, JVM-2 cells, HH cells, NCEB-1 cells and/or HDLM-2 cells, when tested using flow cytometry, for example as described in the Examples herein,

b) is capable of mediating concentration-dependent cytotoxicity of CD30-expressing human tumor cells when using e.g., purified PBMCs, ADCC effector cells or T cells as effector cells when assayed as described in the Examples herein,

c) is capable of mediating concentration-dependent cytotoxicity of one or more human CD30-expressing tumor cell lines selected from the group consisting of: SU-DHL-1 cells, L-428 cells, KM-H2 cells, SUP-M2 cells, KI-JK cells and HDLM-2 cells, when using e.g., purified PBMCs or T cells as effector cells when assayed as described in the Examples herein,

d) is capable of inducing proliferation of T cells in vitro in the presence of CD30-expressing human tumor cells; e.g. when assayed as described in the Examples herein,

e) is capable of activating T cells in vitro in the presence of one or more CD30-expressing human tumor cell lines selected from the group consisting of: SU-DHL-1 cells, L-428 cells, KI-JK cells, and HDLM-2 cells, when assayed as described in the Examples herein,

f) is capable of inducing a dose-dependent production in vitro by T cells of cytokines and Granzyme B, when assayed as described in the examples herein,

g) is capable of not resulting in fratricide of T cells even though CD30 is expressed on a subpopulation of activated T cells, when assayed as described in the examples herein, and/or

h) is capable of inducing T cell mediated cytotoxicity even in the presence of sCD30, when assayed as described in the examples herein.

In one embodiment, the antibody as described herein induces T-cell mediated cytotoxicity with an EC_{50} concentration below 0.050 μg/ml, such as below 0.045 μg/ml, like below 0.040 μg/ml, such as below 0.035 μg/ml, like below 0.030 μg/ml, such as below 0.025 μg/ml, like below 0.020 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry.

In a further embodiment, the antibody as described herein has a maximum lysis when inducing T-cell mediated cytotoxicity above 80%, such as above 85%, like above 90%, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry.

In one embodiment, the antibody as described herein induces $CD4^+$ T cell activation having an EC_{50} concentration of CD25 expression below 0.05 μg/ml, such as below 0.045 μg/ml, like below 0.04 μg/ml, such as below 0.035 μg/ml, like below 0.03 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow

cytometry. In a further embodiment, the antibody as described herein induces $CD8^+$ T cell activation having an EC_{50} concentration of CD25 expression below 0.005 μg/ml, such as below 0.001 μg/ml, like below 0.0009 μg/ml, such as below 0.0008 μg/ml, like below 0.0007 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry.

In one embodiment, the antibody as described herein induces $CD4^+$ T cell activation having an EC_{50} concentration of CD69 expression below 0.004 μg/ml, such as below 0.003 μg/ml, like below 0.002 μg/ml, such as below 0.001 μg/ml, like below 0.0009 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry. In a further embodiment, the antibody as described herein induces $CD8^+$ T cell activation having an EC_{50} concentration of CD69 expression below 0.0035 μg/ml, such as below 0.0030 μg/ml, like below 0.0025 μg/ml, such as below 0.0020 μg/ml, like below 0.0015 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry.

In one embodiment, the antibody as described herein induces $CD4^+$ T cell activation having an EC_{50} concentration of PD-1 expression below 0.01 μg/ml, such as below 0.008 μg/ml, like below 0.007 μg/ml, such as below 0.006 μg/ml, like below 0.005 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry. In a further embodiment, the antibody as described herein induces $CD8^+$ T cell activation having an EC_{50} concentration of PD-1 expression below 0.007 μg/ml, such as below 0.006 μg/ml, like below 0.005 μg/ml, such as below 0.004 μg/ml, like below 0.003 μg/ml, when assayed as described in the Examples herein, such as using L-428 tumor cells and measuring by flow cytometry.

The assays may be performed as commonly known to the skilled person and may optionally, comprise a ratio of effector cells to target cells (E:T) of 4:1 and/or optionally, an incubation period of 72 hrs. The assays could for example be performed by providing tumor cells, preferably labelled tumor cells, such as L-428 tumor cells; adding the antibody as described herein, preferably in a serial dilution; providing T cells, preferably labelled T cells, in a ratio of effector to target being E:T of 4:1 and incubating for 72 hrs; optionally, staining for relevant parameters such as CD4, CD8, CD25, CD69 and/or PD-1, and finally analysing the cells using flow cytometry like FACS. % live cells and/or T cell proliferation/activation may be calculated from the data obtained by the analysis as commonly known to the persons skilled in the art.

Production of antibodies of the invention

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 multispecific,

such as 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.

As mentioned, 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 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 CD3xCD30 antibodies of the present invention includes methods described in WO2011131746 and WO13060867 (Genmab) comprising the following steps:

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 CD30 antibody and the second antibody is a CD3 antibody, or vice versa;
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
d) obtaining said bispecific CD3xCD30 antibody.

Similarly, the invention relates to a method for producing a multispecific, such as a bispecific, antibody according to the invention, comprising

a) providing a first homodimeric antibody comprising the CD30 binding region as described herein, and a second homodimeric antibody comprising the CD3 binding region as described herein, wherein said antibodies comprise an Fc region and optionally contain further features described herein,
wherein the sequences of the first and second CH3 regions of the first and second antibodies are different and are such that the heterodimeric interaction between the first and second CH3 regions is stronger than each of the homodimeric interactions of the first and second CH3 regions;
b) incubating the first antibody together with the second antibody under reducing conditions sufficient to allow the cysteines in the hinge regions to undergo disulfide-bond isomerization; and
c) obtaining a heterodimeric multispecific antibody of the invention as described herein, comprising the first immunoglobulin heavy chain and the first immunoglobulin light chain of the first antibody and the second immunoglobulin heavy chain and the second immunoglobulin light chain of the second 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 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 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 preferred embodiment, the reducing agent is 2-mercaptoethylamine. 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.

In a further aspect, the invention relates to a monospecific antibody comprising

(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and second Fc polypeptide further comprise a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R, wherein the amino acid positions are as defined by Eu numbering.

In one embodiment, the first heavy chain variable region comprises the sequence set forth in SEQ ID NO:13 and the first light chain variable region comprises the sequence set forth in SEQ ID NO:14.

In a further aspect, the invention relates to a monospecific antibody comprising

(i) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and second Fc polypeptide further comprise a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R, wherein the amino acid positions are as defined by Eu numbering.

In one embodiment, X in SEQ ID NO:9 is an H. In another embodiment, X in SEQ ID NO:9 is a G. In one embodiment, the second heavy chain variable region comprises the sequence set forth in SEQ ID NO:15 and the second light chain variable region comprises the sequence set forth in SEQ ID NO:16.

In one embodiment, the above-mentioned monospecific antibodies are full-length antibodies. Preferably, 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 heavy chain has been substituted, wherein preferably, the amino acid in the position corresponding to F405 is L or the amino acid in the position corresponding to K409 is R.

In a further aspect, the invention relates to a method for producing a multispecific antibody, comprising

a) providing a first monospecific CD30 antibody as described herein above and a second antibody comprising

(i) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and a substitution of an amino acid corresponding to the amino acid at position D265 to A in a human IgG1 heavy chain

or

providing a second monospecific CD3 antibody as described herein above and a first antibody comprising

(i) a CD30 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and a substitution of an amino acid corresponding to the amino acid at position D265 to A in a human IgG1 heavy chain

wherein the sequences of the first and second CH3 regions of the first and second antibodies are different and are such that the heterodimeric interaction between the first and second CH3 regions is stronger than each of the homodimeric interactions of the first and second CH3 regions; wherein preferably the amino acid in the position corresponding to F405 is L in the first CH3 region and the amino acid in the position corresponding to K409 is R in the second CH3 region, or vice versa,

b) incubating the first antibody together with the second antibody under reducing conditions sufficient to allow the cysteines in the hinge regions to undergo disulfide-bond isomerization; and

c) obtaining the multispecific antibody comprising the first immunoglobulin heavy chain and the first immunoglobulin light chain of the first antibody and the second immunoglobulin heavy chain and the second immunoglobulin light chain of the second antibody.

In one embodiment, the present invention relates to a method for producing a multispecific antibody as described herein, comprising

a) providing a first monospecific CD30 antibody as described herein and a second antibody comprising

(i) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and a substitution of an amino acid corresponding to the amino acid at position D265 to A in a human IgG1 heavy chain,

wherein the sequences of the first and second CH3 regions of the first and second antibodies are different and are such that the heterodimeric interaction between the first and second CH3 regions is stronger than each of the homodimeric interactions of the first and second CH3 regions; wherein preferably the amino acid in the position corresponding to K409 is R in the first CH3 region and the amino acid in the position corresponding to F405 is L in the second CH3 region,

b) incubating the first antibody together with the second antibody under reducing conditions sufficient to allow the cysteines in the hinge regions to undergo disulfide-bond isomerization; and

c) obtaining the multispecific antibody comprising the first immunoglobulin heavy chain and the first immunoglobulin light chain of the first antibody and the second immunoglobulin heavy chain and the second immunoglobulin light chain of the second antibody.

The present invention further relates to a multispecific antibody obtained by a method as described herein.

In the above methods, the steps of providing a first or second homodimeric antibody capable of binding to CD30 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,
- obtaining said antibody or said antibodies, thereby providing said antibody or said antibodies.

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

The Fc regions of the first and second homodimeric 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 homodimeric antibodies are of the IgG1 isotype. In another embodiment, one of the Fc regions of said homodimeric 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 homodimeric starting antibodies has been engineered to not bind Protein A, thus allowing to separate the heterodimeric antibody from the homodimeric starting antibody by passing the product over a protein A column, removing the flow-through and eluting the heterodimeric antibody from the protein A column, to obtain a purified heterodimeric antibody composition.

As described above, the sequences of the first and second CH3 regions of the homodimeric starting antibodies may be different and 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 CD3xCD30 antibody can be obtained at high yield using the above method of the invention on the basis of two homodimeric starting antibodies which bind CD30 and CD3, 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.

The multispecific, such as bispecific, antibodies of the invention may also be obtained by co-expression of constructs encoding the first and second polypeptides in a single cell.

Thus, in a further aspect, the invention relates to a nucleic acid construct, or a combination of nucleic acid constructs, encoding a multispecific antibody according to the invention and to an expression vector, or a combination of expression vectors, comprising such (a) nucleic acid construct(s).

Furthermore, the invention relates to a recombinant host cell capable of producing the multispecific antibody according to the invention, wherein the host cell comprises one or more nucleic acid constructs encoding the multispecific antibody according to the invention.

Accordingly, the invention also relates to a method for producing a multispecific antibody according to the invention, comprising

(i) culturing a recombinant host cell of the invention under conditions wherein the antibody is produced, and

(ii) isolating the produced multispecific antibody from the culture.

In one embodiment, said method comprises the following steps:

a) providing a first nucleic-acid construct encoding a first polypeptide comprising a first Fc region and a first antigen-binding region of a first antibody heavy chain, said first Fc region comprising a first CH3 region,

b) providing a second nucleic-acid construct encoding a second polypeptide comprising a second Fc region and a second antigen-binding region of a second antibody heavy chain, said second Fc region comprising a second CH3 region,

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, optionally wherein said first and second nucleic acid constructs encode light chain sequences of said first and second antibodies

c) co-expressing said first and second nucleic-acid constructs in a host cell, and

d) obtaining said heterodimeric protein from the cell culture.

Preferably the encoded amino acid in the position corresponding to F405 is L in the first CH3 region and the encoded amino acid in the position corresponding to K409 is R in the second CH3 region, or vice versa.

Suitable expression vectors, including promoters, enhancers, etc., and suitable host cells for the production of antibodies are well-known in the art. Examples of host cells include yeast, bacterial and mammalian cells, such as Chinese hamster ovary cell (CHO) or human, such as human embryonic kidney (HEK), cells.

The nucleic acid, or one or more nucleic acids, as defined herein can be RNA or DNA. The nucleic acid, or one or more nucleic acids, as defined herein may be for use in expression in mammalian cells. Hence, furthermore the invention provides for a cell or cells, comprising a nucleic acid, or comprising one or more nucleic acids, as defined herein.

The nucleic acid in the context of the present invention may be an expression vector, which may be any suitable vector, including chromosomal, non-chromosomal, and synthetic nucleic acid vectors (a nucleic acid sequence comprising a suitable set of expression control elements). Examples of such vectors include derivatives of SV40, bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, and viral nucleic acid (RNA or DNA) vectors. In one embodiment, a CD30 or a CD3 antibody-encoding nucleic acid is comprised in a naked DNA or RNA vector, including, for example, a linear expression element (as described in for instance Sykes and Johnston, Nat Biotech 17, 355 59 (1997)), a compacted nucleic acid vector (as described in for instance US 6,077, 835 and/or WO 00/70087), a plasmid vector such as pBR322, pUC 19/18, or pUC 118/119, a "midge" minimally-sized nucleic acid vector (as described in for instance Schakowski et al., Mol Ther 3, 793 800 (2001)), or as a precipitated nucleic acid vector construct, such as a CaP04-precipitated construct (as described in for instance WO200046147, Benvenisty and Reshef, PNAS USA 83, 9551 55 (1986), Wigler et al., Cell 14, 725 (1978), and Coraro and Pearson, Somatic Cell Genetics 7, 603 (1981)). Such nucleic acid vectors and the usage thereof are well known in the art (see for instance US 5,589,466 and US 5,973,972).

In one embodiment, the vector is suitable for expression of the CD30 antibody and/or the CD3 antibody in a bacterial cell. Examples of such vectors include expression vectors such as BlueScript (Stratagene), pIN vectors (Van Heeke & Schuster, J Biol Chem 264, 5503 5509 (1989), pET vectors (Novagen, Madison WI) and the like).

An expression vector may also or alternatively be a vector suitable for expression in a yeast system. Any vector suitable for expression in a yeast system may be employed. Suitable vectors include, for example, vectors comprising constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH (reviewed in: F. Ausubel et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley InterScience New York (1987), and Grant et al., Methods in Enzymol 153, 516 544 (1987)).

A nucleic acid and/or expression vector may also comprise a nucleic acid sequence encoding a secretion/localization sequence, which can target a polypeptide, such as a nascent polypeptide chain, to the periplasmic space or into cell culture media. Such sequences are known in the art, and include secretion leader or signal peptides. The nucleic acid and/or expression vector may comprise any suitable elements facilitating expression, i.e. transcription and/or translation of the nucleic acid such that the components of the (bispecific) antibodies are

expressed. The nucleic acid and/or vector be associated with any suitable promoter, enhancer, and other expression-facilitating elements. Examples of such elements include strong expression promoters (e. g., human CMV IE promoter/enhancer as well as RSV, SV40, SL3 3, MMTV, and HIV LTR promoters), effective poly (A) termination sequences, an origin of replication for plasmid product in E. coli, an antibiotic resistance gene as selectable marker, and/or a convenient cloning site (e.g., a polylinker). Nucleic acids may also comprise an inducible promoter as opposed to a constitutive promoter such as CMV IE.

Compositions and (medical) uses

Furthermore, the invention provides for a composition comprising an antibody as defined herein. Preferably, such a composition is a pharmaceutical composition, i.e. the antibody is comprised in a pharmaceutically-acceptable carrier.

A pharmaceutical composition may be formulated in accordance with conventional techniques such as those disclosed in Remington: The Science and Practice of Pharmacy, 19th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 1995. A pharmaceutical composition of the present invention may e.g. include diluents, fillers, salts, buffers, detergents (e. g., a nonionic detergent, such as Tween-20 or Tween-80), stabilizers (e. g., sugars or protein-free amino acids), preservatives, tissue fixatives, solubilizers, and/or other materials suitable for inclusion in a pharmaceutical composition.

The pharmaceutical composition may be administered by any suitable route and mode. In one embodiment that pharmaceutical composition is administered by intravenous or subcutaneous injection or infusion.

The antibody, composition, or pharmaceutical composition in accordance with the invention is preferably for use as a medicament.

The antibody, composition, or pharmaceutical composition in accordance with the invention is preferably for use in the treatment of disease.

In particular, the bispecific antibodies of the invention may be used for the treatment of various forms of cancer.

In one aspect, the present invention provides a method for treating a cancer in a subject, which method comprises administration of a therapeutically effective amount of a multispecific antibody of the present invention. In a further embodiment, the present invention provides a method for treating a disorder involving cells expressing CD30, in a subject, which method comprises administration of a therapeutically effective amount of a multispecific antibody of the present invention.

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

CD30. Expression of CD30 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 CD30 transcript and/or protein. The antibodies as described herein that are capable of binding to human CD30 may be used e.g. in immunostaining and/or FACS analysis or the like.

In one aspect, the invention relates to the multispecific antibody, composition or pharmaceutical composition according to the invention for use in the treatment of Hodgkin's lymphoma or anaplastic large cell lymphoma.

In a further aspect, the invention relates to the multispecific antibody, composition or pharmaceutical composition according to the invention for use in the treatment of Hodgkin's lymphoma (HL) or Non-Hodgkin's lymphoma (NHL).

In one embodiment, the Hodgkin's lymphoma is a classical Hodgkin's lymphoma (cHL),

In one embodiment, the Non-Hodgkin's lymphoma is a T cell Non-Hodgkin's lymphoma (T-NHL) or a B cell Non-Hodgkin's lymphoma (B-NHL). In a further embodiment, the Non-Hodgkin's lymphomas is a T cell Non-Hodgkin's lymphoma (T-NHL).

In a further embodiment, the T cell Non-Hodgkin's lymphoma is a peripheral T-cell lymphoma (PTCL) or a cutaneous T-cell lymphoma (CTCL). In a still further embodiment, the T cell Non-Hodgkin's lymphoma (T-NHL) is anaplastic large cell lymphoma (ALCL). In a still further embodiment, the peripheral T-cell lymphoma (PTCL) is anaplastic large cell lymphoma (ALCL).

In one embodiment, the B cell Non-Hodgkin's lymphoma (B-NHL) is mantle cell lymphoma (MCL).

In an even further embodiment, the Hodgkin's lymphoma (HL) is relapsed and refractory Hodgkin's lymphoma. In a further embodiment, the Hodgkin's lymphoma (HL) is a CD30+ Hodgkin's lymphoma. In a still further embodiment, the Hodgkin's lymphoma (HL) is a relapsed and refractory CD30+ Hodgkin's lymphoma. In an even further embodiment, the Hodgkin's lymphoma (HL) is a relapsed and refractory CD30+ classical Hodgkin's lymphoma.

In an even further embodiment, the Non-Hodgkin's lymphoma (NHL) is relapsed and refractory Non-Hodgkin's lymphoma. In a further embodiment, the Non-Hodgkin's lymphoma (NHL) is a CD30+ Non-Hodgkin's lymphoma. In a still further embodiment, the Non-Hodgkin's lymphoma (NHL) is a relapsed and refractory CD30+ Non-Hodgkin's lymphoma.

In a further embodiment, the multispecific antibody, composition or pharmaceutical composition according to the invention for use in the treatment of Hodgkin's lymphoma (HL) or Non-Hodgkin's lymphoma (NHL) is or has been administered intravenously and/or subcutaneously, preferably subcutaneously.

In a further embodiment, a patient being diagnosed with cancer may be subjected to an assessment of CD30 expression in the cancer cells, and when CD30 is detected, which may be

in the range from low to high, such a patient may be selected for treatment with an antibody in accordance with the invention. However, it may not necessarily be a requirement to include such an assessment in selecting a patient for treatment.

The multispecific antibodies of the present invention have numerous *in vitro* and *in vivo* diagnostic and therapeutic utilities involving the diagnosis and treatment of disorders involving cells expressing CD30. For example, the antibodies can be administered to cells in culture, e.g., *in vitro* or *ex vivo*, or to subjects, e.g., *in vivo*, to treat, prevent and/or to diagnose a variety of disorders. As used herein, the term "subject" is intended to include human and non-human individuals.

In one aspect, the invention relates to a diagnostic composition comprising a multispecific antibody according to any one of the embodiments as disclosed herein.

In one embodiment, the diagnostic composition is a companion diagnostic which is used to screen and select those patients who will benefit from treatment with the multispecific antibody.

Kits

The invention further provides a kit-of-parts 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 for predicting efficacy or anti-tumor activity of said antibody when used in treatment of a patient, the kit comprising an antibody as defined above; and instructions for use of said kit.

In one embodiment, the present invention provides a kit for diagnosis of cancer comprising a container comprising a multispecific CD3xCD30 antibody, and one or more reagents for detecting cross-linking of CD30 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 CD30- and CD3-expressing cells occurs in a sample derived from a patient, upon administration of a multispecific antibody according to any one of the embodiments as disclosed herein, comprising the steps of:

(i) contacting the sample with a multispecific 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 CD30-expressing cells and the CD3- expressing cells; and

(ii) analyzing whether a complex has been formed.

Delivery of nucleic acid constructs and delivery vehicles

In a further aspect, the invention relates to the administration of nucleic acid constructs encoding an antibody of the invention for in vivo expression. For in vivo expression of the nucleic acid encoding the antibody, said nucleic acid typically is administered in a form suitable for the nucleic acid to enter the cells of the subject. Different methods for delivering a nucleic acid for in vivo expression exist and include both methods involving mechanical and chemical means. For example, such methods may involve electroporation or tattooing the nucleic acid onto the skin (Patel et al., 2018, Cell Reports 25, 1982-1993). Other methods suitable for administration of the nucleic acid to a subject involve administration of the nucleic acid in a suitable formulation.

Thus, the present invention also relates to a delivery vehicle comprising a nucleic acid or combination of nucleic acids according to the present invention. In some embodiments said delivery vehicle may be a lipid formulation. The lipids of the formulation may be particle(s), such as a lipid nanoparticle(s) (LNPs). The nucleic acid or combination of nucleic acids of the present may be encapsulated within said particle, e.g. within said LNP. Different lipid formulations suitable for administration of a nucleic acid to a subject for in vivo expression are well known to a person skilled in the art. For example, said lipid formulation may typically comprise lipids, ionizable aminolipids, PEG-lipids, cholesterol or any combination thereof.

Various forms and methods for preparation of lipid formulations suitable for administration of a nucleic acid to a subject for expression of a therapeutic antibody are well known in the art. Examples of such lipid formulations include but are not limited to those described in US20180170866 (Arcturus), EP 2391343 (Arbutus), WO 2018/006052 (Protiva), WO2014152774 (Shire Human Genetics), EP 2 972 360 (Translate Bio), US10195156 (Moderna) and US20190022247 (Acuitas).

Accordingly, in a further aspect, the invention relates to (a) nucleic acid construct(s) according to the invention or a delivery vehicle according to the invention for use as a medicament, preferably for use in the treatment of cancer, such as in the treatment of Hodgkin's lymphoma or anaplastic large cell lymphoma.

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

EXAMPLES

Example 1 - Generation of CD3xCD30 bispecific antibodies by 2-MEA-induced Fab-arm exchange

The following antibodies were used in the examples:

Humanized CD3 antibodies

IgG1-huCD3-H1L1 as described in Example 1 of WO2015/001085 (Genmab). IgG1-huCD3-H1L1 is referred to herein as 'IgG1-huCD3'.

IgG1-huCD3-H1L1-H101G as described in Example 2 of WO2017/009442 (Genmab). IgG1-huCD3-H1L1-H101G is referred to herein as 'IgG1-huCD3-H101G'.

CD30 antibodies

MDX-060, which is also referred to as HuMab 5F11, has been disclosed in WO2003/059282 (Medarex). hAC10 (or SGN-30) has been disclosed in US 8257706 and US20100239571 (Seattle genetics). HRS-3 has been disclosed in WO2016/0177846 (Affimed). HeFi-I, T405, T105, T408, and T215 have been disclosed in WO 2007/040653 (US government & Health).

Antibody expression

Antibody sequences were cloned into pcDNA3.3 expression vectors (Invitrogen, US) and expressed as IgG1, κ or IgG1, λ, with or without Fc-silencing and/or DuoBody® technology amino acid substitutions in the Fc domain (see below). All antibodies were produced under serum-free conditions by co-transfecting relevant heavy and light chain expression vectors in Expi293F™ cells (Thermo Fisher Scientific, US; cat. no. A14527) using ExpiFectamine™ 293 (Thermo Fisher Scientific; cat. no. A14525), essentially as described by the manufacturer.

Generation of bispecific antibodies

Bispecific antibodies were generated in vitro using the DuoBody® platform technology, i.e. 2-MEA-induced controlled Fab-arm exchange (cFAE) 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 generation of bispecific antibodies by this method, IgG1 molecules carrying a single mutation in the CH3 domain were generated: in one parental IgG1 antibody the F405L mutation (i.e. the CD3 antibodies or control, HIV-1 gp120-specific, antibodies), in the other parental IgG1 antibody the K409R mutation (i.e. the CD30 or control antibodies). In addition to these mutations, the parental IgG1 antibodies included substitutions that result in an Fc domain that is unable to interact with IgG Fc receptors (Fc gamma receptors) and/or complement factors such as C1q: L234F, L235E, D265A (FEA; US 2015/0337049) or L234F, L235E, G236R (FER).

The combination of Fc silencing and DuoBody® technology mutations were designated as follows:
L234F, L235E, D265A, and F405L: FEAL
L234F, L235E, D265A, and K409R: FEAR

L234F, L235E, G236R, and K409R: FERR

The heavy chain (HC) and light chain (LC) sequences of the parental antibodies are set forth in the following SEQ ID NOs:

IgG1-huCD3-FEAL:	SEQ ID NO:19 (HC) and SEQ ID NO:20 (LC).
IgG1-huCD3-H101G-FEAL:	SEQ ID NO:35 (HC) and SEQ ID NO:20 (LC).
IgG1-CD30-MDX060-FERR:	SEQ ID NO:17 (HC) and SEQ ID NO:18 (LC).
IgG1-CD30-MDX060-FEAR:	SEQ ID NO:55 (HC) and SEQ ID NO:18 (LC).
IgG1-CD30-hAC10-FEAR:	SEQ ID NO:21 (HC) and SEQ ID NO:22 (LC).
IgG1-CD30-HRS-3-FEAR:	SEQ ID NO:23 (HC) and SEQ ID NO:24 (LC).
IgG1-CD30-HeFi-I-FEAR:	SEQ ID NO:25 (HC) and SEQ ID NO:26 (LC).
IgG1-CD30-T405-FEAR:	SEQ ID NO:27 (HC) and SEQ ID NO:28 (LC).
IgG1-CD30-T105-FEAR:	SEQ ID NO:29 (HC) and SEQ ID NO:30 (LC).
IgG1-CD30-T408-FEAR:	SEQ ID NO:31 (HC) and SEQ ID NO:32 (LC).
IgG1-CD30-T215-FEAR:	SEQ ID NO:33 (HC) and SEQ ID NO:34 (LC).

To generate bispecific antibodies, the two parental antibodies were mixed in equal molar ratios 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. Samples were stored overnight at 4 °C to allow for the re-oxidation of the disulfide bonds and formation of intact bispecific antibodies. The efficacy of cFAE was >95 % as assessed by electrospray ionization mass spectrometry mass spectrometry (ESI-MS) as described by Gramer et al. (MAbs. 2013 Nov 1; 5(6): 962–973.).

Non-binding control antibody b12

IgG1-b12 is a HIV-1 gp120 specific antibody (Barbas, CF. J Mol Biol. 1993 Apr 5; 230(3):812-23) that is used in some of the examples as negative, non-binding control antibody. The sequence of the heavy chain and the sequence of the light chain are included herein as SEQ ID NOs: 36 and 37, respectively (FEAL) or SEQ ID NOs: 38 and 37, respectively (FERR).

Example 2 - CD30 expression in human Hodgkin lymphoma (HL), anaplastic large cell lymphoma (ALCL) and TLL cell lines

CD30 surface expression levels were evaluated in a panel of HL, ALCL and TLL cell lines (Table 4) using quantitative flow cytometry (Human IgG Calibrator kit, Biocytex, cat no. CP010). Cells ($5x10^4$ cells/well) were incubated in polystyrene 96-well round-bottom plates (Greiner bio-one, cat. no. 650180) with 10 µg/mL IgG1-CD30-MDX060-FERR in 50 µL staining buffer (PBS [Lonza, cat. no. BE17-517Q] supplemented with 0.1% bovine serum albumin [BSA, fraction V, Roche, cat, no. 10735086001] and 0.02% NaN3 [Sigma Aldrich, cat no. 13412]) at 4°C for 30 min. In parallel, a standard curve was generated using a Human IgG Calibrator Kit (Biocytex, cat. no. CP010), essentially according to the manufacturer's instructions. Calibration beads, containing a well-defined number of human IgG monoclonal antibodies per bead, were incubated with the same R-PE-conjugated secondary antibody (Jackson ImmunoResearch, UK; cat. no. 109-116-098; 1:500 dilution) at 4°C protected from light for 30 min. Cells and beads were washed in FACS buffer and analyzed by flow cytometry on a FACSCelesta flow cytometer (BD Biosciences, USA). The standard curve obtained using the Human IgG Calibrator Kit was used to interpolate the number of IgG1-CD30-MDX060-FERR antibodies bound per cell (ABC) using GraphPad Prism Software, representing an estimate of the number of CD30 molecules expressed on the cell surface.

Table 4 and 5 show that CD30 expression above the lower limit of quantification (LLOQ) was observed in all cell lines except for SUP-T1.

Table 4: Overview of cell lines and expression of CD30

cell line	tumor type	Catalog	supplier	CD30 expression (ABCs)		
				Median	Range	n
L-540	HL	ACC 72	DSMZ	145,723	61,779-271,304	3
L-428	HL	ACC 197	DSMZ	120,725	102,787-133,437	3
HDLM-2	HL	CRL-2965	ATCC	56,578	51,737-87,841	3
KM-H2	HL	ACC 8	DSMZ	19,720	17,283-29,129	3
NCEB-1	MCL	CRL-3005	ATCC	15,406	11,102-18,333	3
L-1236	HL	ACC 530	DSMZ	4,548	4,054-4,838	3
L-82	ALCL	ACC 597	DSMZ	127,616	70,495-172,019	3
SR-786	ALCL	ACC 369	DSMZ	104,947	69,630-133,464	3
KI-JK	ALCL	ACC 695	DSMZ	124,669	77,610-139,598	3
DEL	ALCL	ACC 338	DSMZ	122,003	55,416-153,484	3

SUP-M2	ALCL	ACC 509	DSMZ	6,129	37,047-93,777	3
SU-DHL-1	ALCL	ACC 356	DSMZ	75,238	72,529-80,697	2
HuT 78	ALCL	TIB-161	ATCC	19,399	20,608-18,189	2
SUP-T1	TLL	CRL-1942	ATCC	<LLOQ[a]	-	3

Table 5: Overview of cell lines and average expression of CD30

cell line	**tumor type**	**Catalog**	**supplier**	**CD30 expression (ABCs)**		
				Average	**Range**	**n**
DEL	ALCL	ACC 338	DSMZ	110,301	55,416 – 153,484	3
DL-40	ALCL	JCRB1337	JCRB	98,174	60,392 – 156,229	3
HDLM-2	HL	CRL-2965	ATCC	64,510	51,737 – 87,841	3
HH	CTCL	ACC 707	DSMZ	161,740	122,598 – 206,774	3
HuT 78	ALCL	TIB-161	ATCC	19,399	20,608-18,189	2
JVM-2	MCL	ACC 12	DSMZ	25,842	12,644 – 43,081	3
KARPAS-299	ALCL	06072604	PHE	91,426	77,730-106,058	3
KI-JK	ALCL	ACC 695	DSMZ	118,794	77,610 – 139,598	3
KM-H2	HL	ACC 8	DSMZ	22,044	17,283 – 29,129	3
L-1236	HL	ACC 530	DSMZ	4,480	4,054 – 4,838	3
L-428	HL	ACC 197	DSMZ	117,743	102,787 – 133,437	3
L-540	HL	ACC 72	DSMZ	159,602	61,779 – 271,304	3
L-82	ALCL	ACC 597	DSMZ	127,616	70,495 – 172,019	3
NCEB-1	MCL	CRL-3005	ATCC	14,947	11,102 – 18,333	3
SR-786	ALCL	ACC 369	DSMZ	104,497	69,630 – 133,464	3
SU-DHL-1	ALCL	ACC 356	DSMZ	76,613	72,529-80,697	2
SUP-M2	ALCL	ACC 509	DSMZ	66,129	37,047 – 93,777	3
SUP-T1	TLL	CRL-1942	ATCC	<LLOQ[a]	-	3

[a] <LLOQ, below lower limit of quantification (3300 ABCs)

Example 3 - Binding of CD3xCD30 bispecific antibodies to human Hodgkin lymphoma (HL) and non-Hodgkin's lymphoma (NHL) such as anaplastic large cell lymphoma (ALCL) cells

Binding of CD3xCD30 bispecific antibodies to two CD30-expressing human tumor cell lines SU-DHL-1 (ALCL; ATCC, cat. no. ACC 356) and HDLM-2 (HL; ATCC, cat. no. CRL-2965) was analyzed by flow cytometry.

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 (range 0.0046 to 10 µg/mL in 3-fold dilution steps) in 50 µL staining buffer at 4°C for 30 min. After washing twice in staining buffer, cells were incubated with 50 µL secondary antibody at 4°C for 30 min. As a secondary antibody, R-PE-conjugated goat-anti-human IgG (Jackson ImmunoResearch, UK; cat. no. 109-116-098) diluted 1:400 in staining buffer was used. Next, cells were washed twice in staining buffer, resuspended in 100 µL staining buffer supplemented with TO-PRO-3 iodide (Thermo Fisher Scientific; cat. no. T3605; 1:8000 dilution) and analyzed on a FACSCelesta flow cytometer (BD Biosciences, USA). Live cells were gated based on FSC/SSC and absence of TOPRO-3 staining. Binding curves were analyzed by non-linear regression of log-transformed data (sigmoidal dose-response with variable slope, four parameters) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

Results

Figure 1 shows dose-response binding curves of the CD3xCD30 bispecific antibodies bsG1-huCD3-FEALxCD30-MDX060-FEAR (A), bsG1-huCD3-FEALxCD30-hAC10-FEAR (B), bsG1-huCD3-FEALxCD30-HRS-3-FEAR (C), BsG1-huCD3-FEALxCD30-HeFi-I-FEAR (D), bsG1-huCD3-FEALxCD30-T405-FEAR (E), bsG1-huCD3-FEALxCD30-T105-FEAR (F), BisIgG1-huCD3-FEALxCD30-T408-FEAR (G) and bsG1-huCD3-FEALxCD30-T215-FEAR (H) to SU-DHL-1 (left panels) and HDLM-2 (right panels) tumor cells.

At a concentration of 1.11 µg/mL, bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-hAC10-FEAR, bsG1-huCD3-FEALxCD30-HRS-3-FEAR, and bsG1-huCD3-FEALxCD30-T105-FEAR displayed similar binding when compared the binding of the monospecific, bivalent CD30 parental antibodies IgG1-CD30-MDX060-FEAR, IgG1-CD30-hAC10-FEAR, IgG1-CD30-HRS-3-FEAR and IgG1-CD30-T105-FEAR (Figure 1I).

In contrast, at a concentration of 1.11 µg/mL, the binding of bsG1-huCD3-FEALxCD30-T405-FEAR, bsG1-huCD3-FEALxCD30-T408-FEAR and bsG1-huCD3-FEALxCD30-T215-FEAR was lower than binding of the monospecific, bivalent CD30 parental antibodies IgG1-CD30-T405-FEAR, IgG1-CD30-T408-FEAR and IgG1-CD30-T215-FEAR to SU-DHL-1 and HDLM-2 cells (Figure 1I).

Overall, binding of bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-hAC10-FEAR, bsG1-huCD3-FEALxCD30-HRS-3-FEAR, BsG1-huCD3-FEALxCD30-HeFi-I-FEAR and bsG1-huCD3-FEALxCD30-T105-FEAR at a concentration of 1.11 µg/mL was higher than binding of bsG1-huCD3-FEALxCD30-T405-FEAR, bsG1-huCD3-FEALxCD30-T408-FEAR and bsG1-huCD3-FEALxCD30-T215-FEAR at the same concentration (Figure 1I).

The negative control antibody BsG1-huCD3-FEALxb12-FEAR that was included in these experiments did not show binding to SU-DHL-1 and HDLM-2 cells, indicating that neither SU-DHL-1 nor HDLM-2 cells express CD3.

In conclusion, CD30 antibody clones T405, T408 and T215 showed reduced binding in monovalent compared to bivalent format, while clones MDX060, hAC10, HRS-3 and T105 showed efficient binding to CD30-expressing tumor cells in both monovalent and bivalent format.

Figure 2 shows dose-response binding curves of bsG1-huCD3-FEALxCD30-MDX060-FERR to (A) HDLM-2 (HL), (B) L-428 (HL), (C) DEL (ALCL), and (D) KI-JK (ALCL) cells. BsG1-huCD3-FEALxCD30-MDX060-FERR displayed similar maximum binding when compared to the monospecific, bivalent CD30 parental antibody IgG1-CD30-MDX060-FERR. Negative control antibodies bsG1-huCD3-FEALxb12-FEAR, IgG1-huCD3-FEAL, and IgG1-b12-FEAL did not show binding to any of these cell lines, indicating that these cells do not express CD3 on the cell surface. These data confirmed efficient binding of bsG1-huCD3-FEALxCD30-MDX060-FEER to HL and ALCL cell lines.

Table 6 shows EC_{50} values for binding of bsG1-huCD3-FEALxCD30-MDX060-FEAR to HDLM-2, L-428, DEL, and KI-JK cells as evaluated in two independent experiments. EC_{50} values ranged between 0.05 and 0.30 µg/mL.

Table 6: EC_{50} values for binding of bsG1-huCD3xCD30-MDX060

	EC_{50} (µg/mL)	
Cell line	**N=1**	**N=2**
HDLM-2	0.1450	0.1197
L-428	0.1539	0.0705
DEL	0.3018	0.0568
KI-JK	0.1602	0.0735

Figures 8, 9, and 10 show dose-response binding curves of bsG1-huCD3-FEALxCD30-MDX060-FERR to CD30-expressing HL cell lines (**Figure 8**), ALCL cell lines (**Figure 9**), and NHL cell lines (**Figure 10**). In all cell lines, BsG1-huCD3-FEALxCD30-MDX060-FERR displayed similar maximum binding compared with the monovalent control antibody BsG1-b12-FEALxCD30-MDX060-FERR. The monospecific, bivalent CD30 parental antibody IgG1-CD30-MDX060-FERR showed dose-dependent binding to all cell lines, but showed lower maximum binding compared with BsG1-huCD3-FEALxCD30-MDX060-FERR. Negative control antibodies bsG1-huCD3-

FEALxb12-FEAR, IgG1-huCD3-FEAL, and IgG1-b12-FEAL did not show binding to any of these cell lines, indicating that these cells do not express CD3 on the cell surface. These data confirmed efficient binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to HL cell lines, T-NHL such as ALCL and CTCL cell lines; and B-NHL such as MCL cell line.

Table 7 shows EC_{50} values for binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to HL, T-NHL, and B-NHL cell lines as evaluated in 2 or 3 independent experiments. EC_{50} values ranged between 0.12 and 0.35 µg/mL.

Table 7: EC_{50} values for binding of bsG1-huCD3-FEALxCD30-MDX060-FERR

Cell line		**CD30 Binding**		
Name	**Cancer type**	**Average Binding EC50**	**Range Binding EC_{50} (µg/mL)**	**n tests**
L-540	HL	0.3229	0.2020 - 0.4694	3
L-428	HL	0.1743	0.1742 - 0.1744	2
KM-H2	HL	0.1587	0.1405 - 0.1824	3
L-1236	HL	0.2019	0.1628 - 0.2247	3
HDLM-2	HL	0.1674	0.1253 - 0.2206	3
L-82	ALCL	0.1999	0.1845 - 0.2153	2
SR-786	ALCL	0.3279	0.1735 - 0.5499	3
KI-JK	ALCL	0.1914	0.1431 - 0.2582	3
SUP-M2	ALCL	0.1667	0.1336 - 0.2225	3
DL-40	ALCL	0.2364	0.1698 - 0.3472	3
DEL	ALCL	0.2011	0.1430 - 0.2674	3
KARPAS-299	ALCL	0.2015	0.1343 – 0.2686	2
SUP-T1	TLL	0.3458	0.2572 - 0.4344	2
JVM-2	MCL	0.1257	0.1068 - 0.1411	3
NCEB-1	MCL	0.1564	0.1180 - 0.2000	3
HH	CTCL	0.1951	0.1559 - 0.2425	3

Example 4 - Induction of T-cell mediated cytotoxicity and T-cell proliferation in vitro by CD3xCD30 bispecific antibodies

CD3xCD30 bispecific antibodies were tested in an in vitro cytotoxicity assay using CD30-positive tumor cell lines as target cells and T cells as effector cells. As a source of T cells, CD3-positive ADCC effector cells type IV (Clean Cells, Montaigu, France) or purified T cells (as described in Example 5) were used to evaluate CD3-dependent tumor cell kill.

SU-DHL-1 (ALCL), HuT78 (ALCL), HDLM-2 (HL), NCEB-1 (MCL), or L540 (HL) cells were seeded at a density of 10,000 cells/well into polystyrene 96-well round-bottom plates (Greiner bio-one, cat. no. 650180). Effector cells were labelled with 0.5 µM CFSE (carboxyfluorescein succinimidyl ester; Cell Signalling Technology, Danvers, MA; cat. no. C34554) at 37°C for 20 min and added to tumor cells at an E:T ratio = 10:1 (ADCC effector cells) or 7:1 (purified T cells). Serial dilutions of bispecific CD3xCD30, b12xCD30 or CD3xb12 antibodies, or monospecific, bivalent CD30 antibodies were added (final concentration ranging from 10 to 0.041 µg/mL; 3-fold dilutions) and cells were incubated for 72 hours at 37°C. In some experiments, a variant of bsG1-huCD3-FEALxCD30-MDX060-FEAR was used with a CD3 binding arm containing the H101G mutation, which has with reduced affinity for CD3 (WO2017/009442, Genmab). After washing twice in 100 µL staining buffer, cells were resuspended in staining buffer containing TO-PRO-3 iodide (Thermo Fisher Scientific; cat. no. T3605; 1:4000 dilution) and analyzed on a FACSCelesta flow cytometer (BD Biosciences, USA).

The viability of tumor cell samples treated with 5 µM staurosporine (Sigma-Aldrich, US, cat. no. S6942) was set at 0% and the viability of untreated tumor cell samples was set at 100%.

The 'percentage viable cells' was calculated as follows:

% viable cells = ([cell number sample – cell number staurosporine treated target cells]/[cell number untreated target cells – cell number staurosporine treated target cells]) x 100.

CFSE-positive cells were counted as a measure of absolute numbers of T cells to assess T-cell proliferation.

Dose-response curves were analyzed by non-linear regression (sigmoidal dose-response with variable slope) using GraphPad Prism V8 software (GraphPad Software, San Diego, CA, USA).

Results

CD3xCD30 bispecific antibodies were tested in an *in vitro* cytotoxicity assay using the CD30-positive tumor cell lines SU-DHL-1 cells or HDLM-2 cells as target cells and ADCC effector cells type IV cells (Clean Cells, Montaigu, France) as effector cells.

Figure 3 shows that bsG1-huCD3-FEALxCD30-MDX060-FEAR (A), bsG1-huCD3-FEALxCD30-hAC10-FEAR (B), bsG1-huCD3-FEALxCD30-HRS-3-FEAR (C), BsG1-huCD3-

FEALxCD30-HeFi-I-FEAR (D), bsG1-huCD3-FEALxCD30-T405-FEAR (E), bsG1-huCD3-FEALxCD30-T105-FEAR (F), bsG1-huCD3-FEALxCD30-T408-FEAR (G), and bsG1-huCD3-FEALxCD30-T215-FEAR (H) induced dose-dependent T-cell mediated cytotoxicity (shown as decrease in % viable cells) of SU-DHL-1 (left panels) or HDLM-2 (right panels) cells.

The monospecific, bivalent CD30 antibodies IgG1-MDX060-FEAR (A), IgG1-CD30-hAC10-FEAR (B), IgG1-CD30-HRS-3-FEAR (C), IgG1-CD30-HeFi-I-FEAR (D), IgG1-CD30-T405-FEAR (E), IgG1-CD30-T105-FEAR (F), IgG1-CD30-T408-FEAR (G), and IgG1-CD30-T215-FEAR (H) did not induce T-cell mediated cytotoxicity. Control antibody bsG1-huCD3-FEALxb12-FEAR also did not induce T-cell mediated cytotoxicity of SU-DHL-1 or HDLM-2 cells.

In addition, CD3xCD30 bispecific antibodies were tested in an *in vitro* cytotoxicity assay using different MCL, ALCL and HL cell lines as target cells and purified T cells or ADCC effector cells type IV cells as effector cells. **Figure 4A-B** show that T-cell mediated cytotoxicity of SU-DHL-1, HuT78, or NCEB-1 cells induced by bsG1-huCD3-FEALxCD30-MDX060-FEAR is more potent compared to a variant of this antibody with a CD3 binding arm with reduced affinity (bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR). Similar maximum T-cell mediated cytotoxicity of HDLM-2 cells was induced by bsG1-huCD3-FEALxCD30-MDX060-FEAR and bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR (**Figure 4B**). Incubation with bivalent, monospecific antibodies IgG1-huCD3-FEAL or IgG1-b12-FEAR, which were included as controls, did not induce cytotoxicity in these cell lines. Potent and similar T-cell mediated cytotoxicity of L540 cells was induced by bsG1-huCD3-FEALxCD30-MDX060-FEAR and bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR at the lowest concentration tested (0.014 µg/mL; **Figure 4C**). Control antibodies bsG1-b12-FEALxCD30-MDX060-FEAR or IgG1-MDX060-FEAR did not induce T-cell mediated cytotoxicity of L540 cells.

In conclusion, bsG1-huCD3-FEALxCD30-MDX060-FEAR induced potent killing of various CD30-expressing MCL, ALCL and HL tumor cell lines. BsG1-huCD3-FEALxCD30-MDX060-FEAR that contains an H at position 101 of the VH CDR3 of the CD3 arm is more potent in killing CD30-expressing MCL and ALCL tumor cells compared to the variant with the lower-affinity CD3 arm that contains G at position 101.

In the cytotoxicity assays using HDLM-2 cells (left panel) or NCEB-1 cells (right panel) as target cells, the number of CFSE-positive cells were evaluated as a measure of absolute T-cell counts. **Figure 4D** shows that T-cell mediated cytotoxicity of HDLM-2 and NCEB-1 cells induced by bsG1-huCD3-FEALxCD30-MDX060-FEAR or bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR (see **Figure 4B**) was associated with a dose-dependent increase in T-cell count. Generally, similar T-cell numbers were counted after incubation with bsG1-huCD3-FEALxCD30-MDX060-

FEAR or bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR in this assay. Reduced T-cell numbers were noted in co-cultures with NCEB-1 cells incubated with bsG1-huCD3-FEALxCD30-MDX060-FEAR at concentrations higher than 1 µg/mL. Control antibody IgG1-b12-FEAL did not affect T-cell counts in these experiments.

In conclusion, BsG1-huCD3-FEALxCD30-MDX060-FEAR induced T-cell proliferation in the presence of various CD30-expressing ALCL, HL and MCL tumor cell lines.

Example 5 - Binding of CD3xCD30 bispecific antibodies to human, cynomolgus, or rhesus monkey CD30 expressed in Expi293F cells

Binding of bispecific CD3xCD30 antibodies and monospecific, bivalent CD30 antibodies to the plasma membrane of Expi293 cells transiently transfected with human CD30 or cynomolgus monkey CD30 was analyzed by flow cytometry.

Transient expression of human, cynomolgus or rhesus monkey CD30 in HEK-293F or HEK-293 cells

The following codon-optimized constructs for expression of various full-length CD30 variants were generated: human (Homo sapiens) CD30 (huCD30; Uniprot accession no. P28908), cynomolgus monkey (Macaca fascicularis) CD30 (mfCD30; Uniprot accession no. A0A2K5VW07) (SEQ ID NO:40) and rhesus monkey (Macaca mulatta) CD30 (mmCD30; Uniprot accession no. A0A1D5RK03) (SEQ ID NO:41). The constructs contained suitable restriction sites for cloning and an optimal Kozak (GCCGCCACC) sequence (Kozak, M., Gene 1999;234(2):187-208). The full-length human CD30, cynomolgus and rhesus monkey CD30 codon-optimized constructs were cloned in the mammalian expression vector pcDNA3.3 (Invitrogen) and expressed using the Expi293F expression platform (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. A14527) essentially as described by the manufacturer. In another set of experiments, the full-length human CD30 or cynomolgus monkey CD30 constructs were expressed in HEK-293 cells.

Binding of CD3xCD30 bispecific antibodies to human, cynomolgus monkey or rhesus monkey CD30 expressed in Expi293 cells

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 0.005 to 10 µg/mL in 3-fold dilution steps) in 100 µL 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-PE-conjugated goat-anti-human IgG (Jackson ImmunoResearch, UK; cat. no. 109-116-098) diluted 1:400 in staining buffer was used. Cells were washed twice in staining buffer, resuspended in 30 µL staining buffer containing 0.4%

EDTA 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 V9.0.0 software (GraphPad Software, San Diego, CA, USA).

Binding of CD3xCD30 bispecific antibodies to human, cynomolgus monkey or rhesus monkey CD30 expressed in HEK293 cells

Cells ($3x10^4$ cells/well) were incubated in polystyrene 96-well round-bottom plates (Thermo Scientific, cat. no. 163320) with serial dilutions of antibodies (ranging from 0.0002 to 50 μg/mL in 4-fold dilution steps) in 50 μL 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-PE-conjugated goat-anti-human IgG (Jackson ImmunoResearch, UK; cat. no. 109-116-098) diluted 1:200 in staining buffer was used. Cells were washed twice in staining buffer, resuspended in 30 μL staining buffer containing 0.4% EDTA and the ToPro-3 viability marker (Invitrogen, cat. no. T3605) diluted 1:10,000. Cells were 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 V9.0.0 software (GraphPad Software, San Diego, CA, USA).

Binding of CD3xCD30 bispecific antibodies to human T cells or cynomolgus monkey PBMCs

Cynomolgus monkey PBMCs (Tebu-Bio, The Netherlands; cat. no. PBMCMFA-10) or purified human T cells were plated in polystyrene 96-well round-bottom plates. T cells were derived from 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 manufacturer's instructions. Cells ($3x10^4$ cells/well) were incubated with serial dilutions (ranging from 0.0001 to 10 μg/mL in 3-fold dilution steps) of antibodies IgG1-CD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-MDX060-FEAR, and bsG1-huCD3-FEALxb12-FEAR in 50 μL staining buffer at 4°C for 30 min. After washing twice in staining buffer, cells were incubated in 50 μL secondary R-PE-conjugated goat-anti-human IgG antibody at 4°C for 30 min (1:400 dilution). After washing twice in staining buffer, T cells were stained for T-cell markers CD3 (1:100; Miltenyi biotec, clone 10D12, conjugated to APC), CD4 (1:50; eBioscience, clone OKT4, conjugated to APC-Cy7), CD8 (1:100; Biolegend, clone RPA-T8, conjugated to AF700) and T-cell activation markers CD69 (1:50; BD Biosciences, clone AB2439, conjugated to FITC), CD25 (1:50; eBioscience, clone BC96, conjugated to PE-Cy7) and CD279/PD1 (1:50; BD Biosciences, clone AEH12.2H7, conjugated to BV605). Single stained samples with Ultracomp beads (5 μL; Invitrogen, cat. no. 01-2222-42) were used for compensation adjustments of the flow cytometer. After 30 min of incubation at 4°C, cells were washed twice with staining buffer, resuspended in

100 µL staining buffer and analyzed using a FACS Fortessa (BD Biosciences). Data were processed using FlowJo (BD Biosciences).

Results

CD30-targeting bispecific antibodies bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR and bsG1-b12-FEALxCD30-MDX060-FEAR showed no binding to wild-type Expi293F cells (**Figure 5A**) but displayed dose-dependent binding to Expi293F cells transfected with huCD30 (**Figure 5B**) or mfCD30 (**Figure 5C**). Binding of these bispecific antibodies was comparable to binding of the monospecific, bivalent CD30 antibody IgG1-CD30-MDX060-FEAR. As expected, the negative control antibody bsG1-huCD3-FEALxb12-FEAR showed no binding to wild type or huCD30- or mfCD30-transfected Expi293F cells. Similarly, bsG1-huCD3-FEALxCD30-MDX060-FERR showed dose-dependent binding to HEK293 cells transfected with huCD30 (**Figure 11A**) or mfCD30 (**Figure 11B**), the negative control antibody bsG1-huCD3-FEALxb12-FERR showed no binding to huCD30- or mfCD30-transfected HEK293 cells. This indicates that the CD30-targeting antibody MDX060 efficiently binds to both human and cynomolgus monkey CD30 expressed in HEK cells in a bivalent as well as a monovalent format.

Figure 5D shows that the CD3xCD30 bispecific antibody bsG1-huCD3-FEALxCD30-MDX060-FEAR and control bispecific antibody bsG1-huCD3-FEALxb12-FEAR efficiently bound to primary human and cynomolgus monkey T cells. This shows that these CD3-targeting bispecific antibodies efficiently bind to both human and cynomolgus monkey CD3 endogenously expressed on T cells. The IgG1-CD30-MDX060 bivalent, parental antibody did not bind to human or cynomolgus monkey T cells, indicating that CD30 was not expressed on these cells.

Binding of the panel of CD3xCD30 bispecific antibodies and the monospecific parental CD30 antibodies to Expi293F cells transfected with huCD30 or mmCD30 was evaluated by flow cytometry. The CD3xCD30 bispecific antibodies bsG1-huCD3-FEALxCD30-MDX060-FEAR (**Figure 6A**), bsG1-huCD3-FEALxCD30-hAC10-FEAR (**Figure 6B**), bsG1-huCD3-FEALxCD30-HRS-3-FEAR (**Figure 6C**), bsG1-huCD3-FEALxCD30-T405-FEAR (**Figure 6E**), bsG1-huCD3-FEALxCD30-T105-FEAR (**Figure 6F**), bsG1-huCD3-FEALxCD30-T408-FEAR (**Figure 6G**), and bsG1-huCD3-FEALxCD30-T215-FEAR (**Figure 6H**) showed equal binding to cells expressing huCD30 or mmCD30. Similarly, the parental, monospecific CD30 antibody clones showed equal binding to cells expressing huCD30 or mmCD30. In contrast, bsG1-huCD3-FEALxCD30-HeFi-I-FEAR and the parental, monospecific CD30 antibody IgG1-CD30-HeFi-I_FEAR showed binding to huCD30, but not to mmCD30 (**Figure 6D**).

Example 6 – Assessment of conformational stability of monospecific and bispecific non-activating antibody variants by DSF analysis

Protein stability characteristics of bivalent monospecific CD30, CD3, and bispecific CD3xCD30 IgG1 antibody variants harboring non-activating mutations in the constant heavy chain region were assessed using differential scanning fluorimetry (DSF).

Samples of IgG1-CD30-MDX060-FEAR, IgG1-CD30-MDX060-FERR, IgG1-huCD3-FEAL and BsG1-huCD3-FEALxCD30-MDX060-FERR were formulated in PBS pH 7.4 at a concentration of approximately 1 mg/mL.

To assess conformational stability, DSF was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad) capable of detecting changes in fluorescence intensity caused by binding of the extrinsic dye Sypro-Orange (5000x concentrate in DMSO, Cat # S5692, Sigma-Aldrich) to hydrophobic regions exposed upon unfolding of IgG. Sypro-Orange was diluted 320-fold in PBS pH 7.4 (Hyclone GE Healthcare). A thermal melt curve can be derived from measuring the increasing fluorescence during controlled, stepwise thermal denaturation of the analyzed IgG. Therefore, duplicate samples of 5 µL of antibody solution (1 mg/mL in PBS;) were added to 20 µL of the diluted Sypro-Orange in PBS pH 7.4 in iQ 96-well PCR plates. Fluorescence was recorded at increasing temperatures ranging from 25 °C to 95 °C, in stepwise increments of 0.5 °C per increment and 15 seconds duration plus the time required to record the fluorescence of all wells. The data was analyzed using Bio-Rad CFX Manager Software 3.0 and melting points were determined from the fluorescence versus temperature graphs by the software.

Results

Figure 7 and **Table 8** show that the melting temperature (T_m) of IgG1-CD30-MDX060-FERR is 69.0°C, which is higher than the T_m of IgG1-CD30-MDX060-FEAR at pH 7.4 (64.5°C). This indicates that IgG1-CD30-MDX060-FERR has higher conformational stability than IgG1-CD30-MDX060-FEAR suggesting that IgG1-CD30-MDX060 containing a FER backbone has a higher conformational stability than IgG1-CD30-MDX060 containing a FEA backbone. The melting temperature of BsG1-huCD3-FEALxCD30-MDX060-FERR was determined to be 64.5°C, which is between the T_m determined for the two parental antibodies IgG1-huCD3-FEAL (62.5°C) and IgG1-CD30-MDX060-FERR (69.0°C).

Table 8: Melting temperatures (T_m) of indicated antibodies as determined by differential scanning fluorimetry (DSF)

Antibody	**T_m (°C)**
IgG1-CD30-MDX060-FEAR	64.5

IgG1-CD30-MDX060-FERR	69.0
IgG1-huCD3-FEAL	62.5
BsG1-huCD3-FEALxCD30-MDX060-FERR	64.5

Example 7 – Simultaneous binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to T cells and tumor cells

Simultaneous binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to tumor cells and naive T cells was studied.

Frozen T cells isolated from healthy donors were thawed and labelled with 0.25 mM Celltrace Violet (Pacific Blue; Invitrogen, cat no. C34557A) at 37°C for 15 min. L-428 tumor cells were labelled with Celltrace FarRed (APC; Invitrogen cat no. C34564A) at 37°C for 15 min and added to T cells at E:T ratio of 1:1. Serial dilutions of bsG1-huCD3-FEALxCD30-MDX060-FERR or control antibodies bsG1-huCD3-FEALxb12-FEAR, bsG1b12FEALxCD30MDX060-FERR, or IgG1-b12-FEAL were added (final concentration ranging from 6×10^{-5} to 10 µg/mL; 3-fold dilutions) and cells were incubated for 2 hours at 4°C. After incubation, viability marker 7-AAD (BD Bioscience, cat. no 559925) was added (100x final dilution) and cells were analyzed on a FACS Celesta flow cytometer (BD Biosciences).

Results

Figure 12 shows that bsG1-huCD3-FEALxCD30-MDX060-FERR induces the formation of $CD3^+CD30^+$ double-positive events (cells expressing both CellTrace Far Red and CellTrace Violet in flow cytometry staining) as a measure for bsG1-huCD3-FEALxCD30-MDX060-FERR-mediated crosslinking of tumor cells to T cells (simultaneous binding). The increase in double-positive events was antibody concentration-dependent and showed a bell-shaped curve. No increase in crosslinking of tumor cells and T cells was observed in samples incubated with control antibodies bsG1-huCD3-FEALxb12-FEAR, bsG1b12FEALxCD30MDX060-FERR, or IgG1-b12-FEAL or in samples incubated without antibody (A). Figure 12 B shows simultaneous binding of bsG1-huCD3-FEALxCD30-MDX060-FERR to tumor cells and naive T cells,as detected by the percentage of cells expressing both CellTrace Far Red and CellTrace Violet (B).

These data indicate that bsG1huCD3FEALxCD30-MDX060-FERR can simultaneously bind and crosslink $CD30^+$ tumor cells and $CD3^+$ T cells.

Example 8 – Induction of T-cell mediated cytotoxicity and T-cell activation *in vitro* by CD3xCD30 bispecific antibodies

T-cell mediated cytotoxicity of Karpas-299 tumor cells and associated T-cell activation by a panel of CD3xCD30 bispecific antibodies was evaluated. The following antibodies were evaluated: bsG1-huCD3-FEALxCD30-MDX060-FERR, bsG1-huCD3-FEALxCD30-MDX060-FEAR, bsG1-huCD3-FEALxCD30-hAC10-FEAR, bsG1-huCD3-FEALxCD30-HRS-3-FEAR, bsG1-huCD3-FEALx CD30-HeFi-I-FEAR, bsG1-huCD3-FEALxCD30-T105-FEAR, bsG1-huCD3-FEALxCD30-T405-FEAR, bsG1-huCD3-FEALx CD30-T408-FEAR, and bsG1-huCD3-FEALx CD30-T215-FEAR.

T cells were obtained from healthy human donor buffy coats (Sanquin, Amsterdam, The Netherlands) and isolated using a RosetteSep™ human T-cell enrichment cocktail (Stemcell Technologies, France, cat. no. 15061) according to the manufacturer's instructions. T cells were labelled with Celltrace Violet (Invitrogen, cat. no. C34557A; final concentration 5 μM) for 15 min at 37°C. In parallel, Karpas-299 tumor cells were labeled with Celltrace FarRed (Invitrogen, cat. no. C34564A; final concentration 2 μM) for 15 min at 37°C. After labeling, 5× the volume of ice cold DBSI was added and incubated for 5 minutes at RT. Cells were pelleted, resuspended in medium, and tumor cells were seeded into 96-well plates (Greiner-bio-one, The Netherlands, cat. no. 655180) at a density of 50,000 cells/well. Serial dilutions of bispecific CD3xCD30 antibodies were added (final concentration ranging from 1,000 to 0.051 ng/mL; 3-fold dilutions) and plates were incubated for 15 min at RT. T cells were added to tumor cells at an effector to target (E:T) ratio of 4:1 and plates were incubated for 72 hours at 37°C. After washing 2 times with PBS/0.1% BSA/0.02% azide (staining buffer), cells were stained for T-cell markers CD4 (1:50; Biolegend, cat. no. 300521, conjugated to Pacific Blue), CD8 (1:100; BD Biosciences, conjugated to FITC) and T cell activation markers CD69 (1:50; Biolegend, cat. no. 310934, conjugated to BV650), CD25 (1:100; Invitrogen, cat. no. 25-0259-42, conjugated to PE-Cy7) and CD279/PD-1 (1:50; Biolegend, cat. no. 329930, 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 twice with staining buffer, and cells were stained with 7-AAD (diluted 1:100 in staining buffer) for 10 min at 4°C. Cells were analyzed using a FACS Celesta (BD Biosciences). Data were processed using FlowJo (BD Biosciences).

Dose-response curves were generated using non-linear regression analysis (sigmoidal dose-response with variable slope) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

Results

Figure 13A and B show that all CD3xCD30 antibodies induce T-cell mediated cytotoxicity of Karpas-299 cells. CD3xCD30 bispecific antibodies generated using CD30 clone MDX060 (bsG1-huCD3-FEALxCD30-MDX060-FERR and bsG1-huCD3-FEALxCD30-MDX060-FEAR) were more effective in killing Karpas-299 cells compared to all other clones tested. In fact, MDX060-based CD3xCD30 bispecific antibodies showed significantly lower IC_{50} values compared to CD3xCD30 bispecific antibodies generated using CD30 clones HRS-3, HeFi-I, T105, T405, T408, or T215 (bsG1-huCD3-FEALxCD30-HRS-3-FEAR, bsG1-huCD3-FEALxCD30-HeFi-I-FEAR, bsG1-huCD3-FEALxCD30-T105-FEAR, bsG1-huCD3-FEALxCD30-T405-FEAR, bsG1-huCD3-FEALxCD30-T408-FEAR, and bsG1-huCD3-FEALxCD30-T215-FEAR; **Figure 13A**). Furthermore, bsG1-huCD3-FEALxCD30-MDX060-FERR and bsG1-huCD3-FEALxCD30-MDX060-FEAR induced higher maximum killing compared to CD3xCD30 bispecific antibodies generated using CD30 clones hAC10, HeFi-I, T405, T408, or T215 (bsG1-huCD3-FEALxCD30-hAC10-FEAR, bsG1-huCD3-FEALxCD30-HeFi-I-FEAR, bsG1-huCD3-FEALxCD30-T405-FEAR, bsG1-huCD3-FEALxCD30-T408-FEAR, and bsG1-huCD3-FEALxCD30-T215-FEAR; **Figure 13B)**. **Figure 13C and D** show that bsG1-huCD3-FEALxCD30-MDX060-FERR and bsG1-huCD3-FEALxCD30-MDX060-FEAR were more effective (lower EC_{50} values) than any of the other CD3xCD30 bispecific antibodies at inducing expression of CD25, as a measure of T-cell activation in $CD4^+$ T cells (**Figure 13C**) or $CD8^+$ T cells (**Figure 13D**). Similar results were observed for expression of PD-1 (**Figure 13E and F**) and CD69 (data not shown). No difference in T-cell-mediated killing or T-cell activation was observed between the two MDX060-based CD3xCD30 bispecific antibodies that contain the FEAR or FERR mutations.

The bispecific antibody bsG1-huCD3xCD30-MX060 was also more effective at inducing T-cell mediated cytotoxicity of L-428 cells in comparison to other panel CD3xCD30 bispecific antibodies tested (data not shown).

The average IC_{50} concentrations and maximum percentage lysis of T-cell mediated cytotoxicity and EC_{50} concentrations of T-cell activation (CD25 expression) induced by the panel of CD3xCD30 bispecific antibodies used in these experiments are summarized in **Table 9**.

In conclusion, these data demonstrated that bsG1huCD3xCD30-MDX060 was more effective than any of the other CD3xCD30 bispecific antibodies evaluated at inducing T-cell mediated cytotoxicity.

Table 9: IC_{50} concentrations and maximum percentage lysis of T-cell mediated cytotoxicity of Karpas-299 cells and EC_{50} concentrations of T-cell activation (CD25 expression) induced by a panel of CD3xCD30 bispecific antibodies.

Antibody	**cytotoxicity**		**T-cell activation (CD25 expression)**		**T-cell activation (PD-1 expression)**	
			CD4+ T cells	**CD8+ T cells**	**CD4+ T cells**	**CD8+ T cells**
	IC_{50} (µg/mL)	maximum lysis (%)	EC_{50} (µg/mL)	EC_{50} (µg/mL)	EC_{50} (µg/mL)	EC_{50} (µg/mL)
	average (SD)	average (SD)	average (SD)	average (SD)	average (SD)	average (SD)
bsG1-huCD3-FEALxCD30-MDX060-FERR	0.029 (0.004)	52.328 (3.929)	0.023 (0.004)	0.020 (0.005)	0.025 (0.006)	0.023 (0.009)
bsG1-huCD3-FEALxCD30-MDX060-FEAR	0.033 (0.004)	53.703 (5.949)	0.024 (0.004)	0.020 (0.005)	0.024 (0.006)	0.027 (0.009)
bsG1-huCD3-FEALxCD30-hAC10-FEAR	0.032 (0.006)	37.765 (5.340)	0.046 (0.011)	0.054 (0.021)	0.056 (0.021)	0.057 (0.031)
bsG1-huCD3-FEALxCD30-HRS-3-FEAR	0.068 (0.011)	47.472 (6.539)	0.089 (0.031)	0.079 (0.031)	0.123 (0.046)	0.112 (0.047)
bsG1-huCD3-FEALxCD30-HeFi-I-FEAR	0.052 (0.011)	42.987 (6.603)	0.069 (0.013)	0.076 (0.016)	0.075 (0.024)	0.083 (0.023)
bsG1-huCD3-FEALxCD30-T105-FEAR	0.042 (0.007)	52.582 (5.344)	0.056 (0.010)	0.060 (0.017)	0.064 (0.021)	0.065 (0.024)
bsG1-huCD3-FEALxCD30-T405-FEAR	0.078 (0.023)	41.667 (4.560)	0.093 (0.027)	0.080 (0.056)	0.138 (0.054)	0.140 (0.126)
bsG1-huCD3-FEALxCD30-T408-FEAR	0.226 (0.112)	41.295 (5.482)	0.172 (0.046)	0.134 (0.077)	0.286 (0.104)	0.220 (0.126)
bsG1-huCD3-FEALxCD30-T215-FEAR	0.077 (0.032)	42.840 (6.464)	0.092 (0.036)	0.105 (0.063)	0.355 (0.476)	0.281 (0.308)

Example 9 – Induction of T-cell mediated cytotoxicity, T-cell proliferation, and T-cell activation *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR

5 T-cell mediated cytotoxicity of tumor cells and associated T-cell proliferation and activation by bsG1-huCD3-FEALxCD30-MDX060-FERR was evaluated in HL and ALCL cell lines.

T cells were obtained from healthy human donor buffy coats (Sanquin, Amsterdam, The Netherlands) and isolated using a RosetteSep™ human T-cell enrichment cocktail (Stemcell Technologies, France, cat. no. 15061) according to the manufacturer's instructions. T cells were labelled with Celltrace Violet (Invitrogen, cat. no. C34557A; final concentration 5 μM) for 15 min at 37°C. In parallel, tumor cells L-428, KI-JK, KM-H2, or SUP-M2 were labeled with Celltrace FarRed (Invitrogen, cat. no. C34564A; final concentration 2 μM) for 15 min at 37°C. After labeling, 5× the volume of ice cold DBSI was added and incubated for 5 minutes at RT. Cells were pelleted, resuspended in medium, and tumor cells were seeded into 96-well plates (Greiner-bio-one, The Netherlands, cat. no. 655180) at a density of 50,000 cells/well. Serial dilutions of bsG1-huCD3-FEALxCD30-MDX060-FERR or control antibodies IgG1-huCD3-FEAL, bsG1-huCD3-FEALxb12-FERR, IgG1-CD30-MDX060-FERR, bsG1-b12-FEALxCD30-MDX060-FERR, IgG1-b12-FEAL were added (final concentration ranging from 1,000 to 0.051 ng/mL; 3-fold dilutions) and plates were incubated for 15 min at RT. T cells were added to tumor cells at an effector to target (E:T) ratio of 4:1 and plates were incubated for 72 hours at 37°C. After washing 2 times with PBS/0.1% BSA/0.02% azide (staining buffer), cells were stained for T-cell markers CD4 (1:50; Biolegend, cat. no. 300521, conjugated to Pacific Blue), CD8 (1:100; BD Biosciences, cat. no. 345772, conjugated to FITC) and T cell activation markers CD69 (1:50; Biolegend, cat. no. 310934, conjugated to BV650), CD25 (1:100; Invitrogen, cat. no. 25-0259-42, conjugated to PE-Cy7) and CD279/PD-1 (1:50; Biolegend, cat. no. 329930, 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 twice with staining buffer and cells were stained with 7-AAD (diluted 1:100 in staining buffer) for 10 min at 4°C. Cells were analyzed using a FACS Celesta (BD Biosciences) and data were processed using FlowJo (BD Biosciences).

The percentage of live target cells was calculated using the following equation:-
% live target cells = (absolute number of live, single Celltrace FarRed-labeled cells in each condition / absolute number of live, single Celltrace FarRed labeled cells in the condition containing only target cells and T cells without adding any antibodies) x 100.

T-cell proliferation was evaluated by gating $CD4^+$ or $CD8^+$ T cells with diluted Celltrace Violet staining. The expansion index was calculated using the proliferation modeling tool from FlowJo. Generation peaks were automatically fitted and expansion index values were calculated according the following equation:

Expansion index = Total amount of cells/amount of cells at start of culture = (G0 + G1 + G2 + G3 + G4 + G5 + G6)/(G0 + G1:2 + G2:4 + G3:8 + G4:16 + G5:32 + G6:64).
Gn = number of cells in generation n peak (with n = 0 to 6).

Dose-response curves were generated using non-linear regression analysis (sigmoidal dose-response with variable slope) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

5 *Results*

Figure 14 shows that bsG1-huCD3-FEALxCD30-MDX060-FERR induced dose-dependent T-cell mediated cytotoxicity in L-428 (HL), KM-H2 (HL), SUP-M2 (ALCL), and KI-JK (ALCL) cell lines *in vitro*. The average IC50 concentrations of T-cell mediated cytotoxicity induced by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 and KI-JK cells are summarized in **Table 10**. No cytotoxicity
10 was observed in cells incubated with control antibodies IgG1-huCD3-FEAL, bsG1-huCD3-FEALxb12-FERR, IgG1-CD30-MDX060-FERR, bsG1-b12-FEALxCD30-MDX060-FERR, IgG1-b12-FEAL or in samples incubated without antibody.

Figures 15, 16, 17, and 18 show that T-cell mediated cytotoxicity of L-428 and KI-JK cells
15 induced by bsG1-huCD3-FEALxCD30-MDX060-FERR was associated with $CD4^+$ and $CD8^+$ T cell proliferation (**Figure 15**) and expression of T-cell activation markers CD69 (**Figure 16**), CD25 (**Figure 17**) and PD-1 (**Figure 18**). The average EC50 concentrations of T-cell proliferation and activation induced by bsG1-huCD3-FEALxCD30-MDX060-FERR in these experiments are summarized in **Table 10**.

20

Hence, bsG1-huCD3-FEALxCD30-MDX060-FERR induced dose-dependent T-cell mediated cytotoxicity in HL and ALCL cell lines *in vitro*, which was associated with T-cell proliferation and activation.

25 Table 10: EC_{50} concentrations of T-cell mediated cytotoxicity induced by bsG1-huCD3-FEALxCD30-MDX060-FERR in L-428 and KI-JK cells and associated T-cell proliferation and activation of $CD4^+$ and $CD8^+$ cells.

Tumor Cell line		**L-428**		**KI-JK**	
Cytotoxicity					
	Average IC50 (µg/mL)	0.002		0.0015	
	Range IC50 (µg/mL)	0.001216 - 0.003439		0.0008858 - 0.001904	
T cell type		$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
T cell proliferation					

PI	Average EC50 (µg/mL)	0.0051	0.00096	0.00606	0.00213
	Range EC50 (µg/mL)	0.003727 - 0.006534	0.0002052 - 0.002255	0.00338 - 0.007501	0.001103 - 0.002715
T cell activation					
CD69	Average CD69 EC50 (µg/mL)	0.00083	0.00121	0.00146	0.00171
	Range CD69 EC50 (µg/mL)	0.0001221 - 0.001647	3.252E-14 - 0.002421	0.0004487 - 0.002086	4.694E-11 - 0.005097
CD25	Average CD25 EC50 (µg/mL)	0.00261	0.00061	0.00599	0.00656
	Range CD25 EC50 (µg/mL)	0.002164 - 0.002898	0.000298 - 0.001006	0.004483 - 0.007791	0.001901 - 0.01562
PD-1	Average PD-1 EC50 (µg/mL)	0.00491	0.00257	0.0112	0.00696
	Range PD-1 EC50 (µg/mL)	0.004387 - 0.005433	0.001302 - 0.003412	0.009616 - 0.01222	0.005845 - 0.009151
n test		3		3	

Example 10 - Induction of cytokine production *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR

Cytokine and Granzyme B production induced by bsG1-huCD3-FEALxCD30-MDX060-FERR was evaluated in supernatants that were collected during an *in vitro* T cell-mediated cytotoxicity experiment using L-428 target cells and healthy donor T cells, as described in example 9. Supernatants were stored at -20°C and thawed for analysis. The concentration of 14 different cytokines (CD40, IFNγ, IL-10, IL-12, IL-13, IL-1b, IL-2, IL-4, IL-6, IL-8, IP-10, MCP-1, PDL-1, TNFα) and Granzyme B were measured using a bead-based multiplex immuno-assay (luminex) custom made by R&D systems.

Results

Increased concentrations were primarily observed for Granzyme B and cytokines IFNγ, IL-13 and TNFα (>2000 pg/mL) in supernatants from co-cultures of L-428 cells and T cells in the presence of bsG1-huCD3-FEALxCD30-MDX060-FERR. A moderate increase was observed for CD40, IL-10, IL-12, IL-1β, IL-2, IL-4, IL6, and IP-10 cytokine concentrations, when compared to the control

antibody IgG1-b12-FEAL. Levels of IL-8, MCP-1, and PDL1 were not modulated compared to the control antibody IgG1-b12-FEAL (**Figure 19**).

Thus, T-cell mediated cytotoxicity and T-cell activation by bsG1-huCD3-FEALxCD30-MDX060-FERR was associated with dose-dependent production of cytokines and Granzyme B.

Example 11 - Induction of T-cell mediated cytotoxicity *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR using purified T cells as effector cells at varying effector to target ratios

To determine the optimal effector to target cell ratio of T cell-mediated tumor cell kill in the presence of bispecific antibody bsG1-huCD3-FEALxCD30-MDX060-FERR, an *in vitro* cytotoxicity assay was performed using the CD30-positive tumor cell line L-428 as target cells and purified T cells as effector cells at varying effector to target cell (E:T) ratios.

T-cell mediated cytotoxicity was evaluated essentially as described in Example 9, except that T cells were added to tumor cells at varying effector to target (E:T) cell ratios of 1:1, 2:1, 4:1 or 8:1.

Results

Figure 20A shows that dose-dependent T-cell mediated cytotoxicity was induced by bsG1-huCD3-FEALxCD30-MDX060-FERR at all E:T ratios, with maximum tumor cell kill (less than 20% viable tumor cells) observed at E:T ratios of 4:1 and 8:1. In line with this, $CD4^+$ and $CD8^+$ T cell proliferation was observed at all E:T cell ratios, most prominently at E:T ratios of 4:1 and 8:1 (**Figure 20B-C**). No specific T-cell mediated cytotoxicity or T-cell proliferation was induced by control antibody bsG1-huCD3-FEALxb12-FERR at any of the E:T ratios tested.

Together, these data indicate that bsG1-huCD3-FEALxCD30-MDX060-FERR-induced T-cell mediated cytotoxicity of L-428 tumor cells in vitro was most effective at E:T ratios of 4:1 and 8:1.

Example 12 - Kinetics of T-cell mediated cytotoxicity and T-cell proliferation *in vitro* by bsG1-huCD3-FEALxCD30-MDX060-FERR

To evaluate the kinetics of T cell-mediated tumor cell kill in the presence of bsG1-huCD3-FEALxCD30-MDX060-FERR, an *in vitro* cytotoxicity assay was performed at varying incubation periods using the CD30-positive tumor cell line L-428 as target cells and purified T cells as effector cells.

T-cell mediated cytotoxicity was evaluated essentially as described in Example 9, except that tumor cell cytotoxicity and T-cell proliferation was evaluated after 24 h, 48 h and 72 h.

Results

Figure 21 shows that bsG1-huCD3-FEALxCD30-MDX060-FERR induced dose-dependent T-cell mediated cytotoxicity after 48 h and 72 h, while no significant T-cell mediated cytotoxicity was observed after 24 h. Dose-dependent $CD4^+$ and $CD8^+$ T cell proliferation was induced by bsG1-huCD3-FEALxCD30-MDX060-FERR after 72 h, while no T-cell proliferation was observed after 24 h or 48 h (**Figure 21B and C**).

Together, these data show that bsG1-huCD3-FEALxCD30-MDX060-FERR induced T-cell mediated cytotoxicity of tumor cells and T-cell proliferation in a time-dependent manner.

Example 13 - Correlation of CD30 expression level with bsG1-huCD3-FEALxCD30-MDX060-FERR-induced T-cell mediated cytotoxicity *in vitro*

T-cell mediated kill by bsG1-huCD3-FEALxCD30-MDX060-FERR of eight CD30 expressing tumor cell lines was determined in an *in vitro* cytotoxicity assay as described in example 9, using an E:T ratio of 4:1. The following cell lines were used: L-428, KM-H2, DEL, KI-JK, KARPAS-299, SUP-M2, NCEB-1, and JVM-2. For these tumor cell lines, CD30 expression levels were evaluated by quantitative flow cytometry as detailed in example 2.

Results

Figure 22 shows that bsG1-huCD3-FEALxCD30-MDX060-FERR induced T-cell mediated cytotoxicity in all cell lines *in vitro* with maximum target cell kill between 68% and 98%. Maximum T-cell mediated tumor cell kill by bsG1-huCD3-FEALxCD30-MDX060-FERR significantly correlated with the level of CD30 expression (**Figure 22A**).

In **Figure 22B** the EC_{50} of T cell-mediated kill in the presence of bsG1-huCD3-FEALxCD30-MDX060-FERR for each cell line is plotted against CD30 expression levels, showing a negative, but nonsignificant, trend.

Thus, these data show a positive correlation between CD30 expression levels and bsG1-huCD3-FEALxCD30-MDX060-FERR-induced maximum T-cell mediated cytotoxicity in vitro.

Example 14 - Fratricide of activated T cells by BsG1-huCD3-FEALxCD30-MDX060-FERR

BsG1-huCD3-FEALxCD30-MDX060-FERR-induced T-cell fratricide of activated-$CD30^+$ T-cells was evaluated in vitro.

A 96-well plate (Greiner-bio-one, The Netherlands, cat. no. 655180) was coated with 100 µL of a solution of 1 µg/mL anti-human CD3 (clone OKT3, Invitrogen, cat. no. 16-0037-85) in PBS. The plate was incubated for 4 hours at 37°C. After removing the antibody solution, wells were washed with 100 µL of PBS. T cells were obtained from healthy human donor buffy coats

(Sanquin, Amsterdam, The Netherlands) and isolated using aRosetteSep™ human T cell enrichment cocktail (Stemcell Technologies, France, cat. no. 15061) according to the manufacturer's instructions. Purified T cells were resuspended in T-cell medium (Roswell Park Memorial Institute [RPMI]-1640 medium with 25 mM HEPES and L-glutamine (Lonza, cat. no. BE12-115F), supplemented with 10% heat-inactivated donor bovine serum with iron (DBSI; Gibco, cat. no. 20731-030) and penicillin/streptomycin (pen/strep; Lonza, cat. no. DE17-603E) at a concentration of $2x10^6$ cells/mL 100 µL of T-cell suspension (containing 200,000 T-cells) was added to each well of the anti-CD3 coated plate. Furthermore, 100 µL of T cell culture medium supplemented with 2 µg/mL anti-CD28 (clone CD28.2, Invitrogen, cat. no. 16-0289-85) and 0.05 µg/mL IL-15 (ThermoFisher, cat. no. PHC9151) was added to each well. T-cells were then incubated for 96 hours at 37°C.

After 96 hours, T cells were collected and resuspended in T-cell medium at a concentration of $2x10^6$ cells/mL. Flow cytometry analysis was performed to measure the expression of CD30 and T-cell activation markers. In brief, an aliquot of cells was washed with PBS/0.1% BSA/0.02% azide (staining buffer), stained with 50 µl of 1000x diluted FVS510 viability dye (BD Biosciences, cat. no. 564406) and incubated at room temperature for 15 minutes. Then the cells were washed with staining buffer, and stained for CD30 (1:50; Biolegend, cat. no. 333906, conjugated to PE), T-cell markers CD4 (1:50; Biolegend, cat. no. 300506, conjugated to FITC), CD8 (1:100; Biolegend, cat. no. 301028, conjugated to AF700) and T-cell activation markers CD69 (1:50; Biolegend, cat. no. 310910, conjugated to APC), CD25 (1:100; Invitrogen, cat. no. 25-0259-42, conjugated to PE-Cy7) and CD279/PD1 (1:50; Biolegend, cat. no. 329924, 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 twice with staining buffer. Cells were analyzed using a FACS Celesta (BD Biosciences) and data were processed using FlowJo (BD Biosciences).-

To assess whether the BsG1-huCD3-FEALxCD30-MDX060-FERR can induce fratricide of activated T cells, stimulated T cells were seeded in a 96-well plate at a density of 200,000 cells/well. Subsequently, 50 µL of BsG1-huCD3-FEALxCD30-MDX060-FERR, or control antibodies, i.e. bsG1-b12-FEALxCD30-MDX060-FERR, bsG1-huCD3-FEALxb12-MDX060-FERR, or IgG1-b12 were added to each well (final concentration ranging from 0.003 to 3.3 µg/mL in 3-fold dilution steps in T-cell medium). Plates were incubated at 37°C for 48 hours.

After washing 2 times with staining buffer, cells were stained with FVS510 viability dye and consequently stained for T-cell markers CD4 and CD8 and T cell activation markers CD69, CD25 and CD279/PD1. Flow cytometric analysis was performed using a FACS Celesta (BD Biosciences) and data were processed using FlowJo (BD Biosciences).

Dose-response curves were generated using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

Results

Figure 23 shows that cell markers CD25 (T-cell activation) **(A)** and CD30 **(B)** were expressed in 54-63% and 21-27% of T cells respectively after 72 hours of incubation. Expression of CD25 and CD30 was further induced after 96 hours of incubation (CD25: 80-83% and CD30: 27-33%). **Figure 23C** shows that increasing doses of BsG1-huCD3-FEALxCD30-MDX060-FERR, bsG1-b12-FEALxCD30-MDX060-FERR, bsG1-huCD3-FEALxb12-MDX060-FERR, or IgG1-b12 were not associated with reduced viability of activated T cells.

Therefore, CD30 expression on a subpopulation of activated T cells did not result in fratricide of T cells upon incubation with BsG1-huCD3-FEALxCD30-MDX060-FERR.

Example 15- Interference of sCD30 with the anti-tumor activity of BsG1-huCD3-FEALxCD30-MDX060-FERR

Shedding of cell surface CD30 and generation of soluble CD30 (sCD30) were evaluated in 17 different hematological $CD30^+$ tumor cell lines.

The concentration of sCD30 in 25µL of undiluted supernatant collected from cell cultures three days after seeding cells in the fresh medium was measured by an ELISA assay for quantitative detection of human CD30, using the "Human sCD30 ELISA Kit" (Invitrogen, cat. no. BMS240), following the manufacturer's instructions.

Results

Figure 24A shows the concentration of sCD30 in the cell culture supernatant. As shown, varying concentrations of sCD30 were detected in the cell culture supernatants from different cell lines. **Figure 24B** shows that the concentration of sCD30 in the cell culture supernatants significantly correlated with CD30 membrane expression levels, as measured by quantitative flow cytometry as described previously in example 2 (Human IgG Calibrator kit, Biocytex, cat no. CP010).

To evaluate whether sCD30 can block potent BsG1-huCD3-FEALxCD30-MDX060-FERR-induced T-cell mediated cytotoxicity, T-cell mediated cytotoxicity by BsG1-huCD3-FEALxCD30-MDX060-FERR was assessed in DEL tumor cells (ALCL), which showed high levels of sCD30 in the supernatant (129 ng/mL). The T-cell mediated cytotoxicity assay was performed as described previously (example 8). **Figure 24C** shows that BsG1-huCD3-FEALxCD30-MDX060-FERR induced potent T-cell mediated cytotoxicity in this cell line, with maximum tumor cell kill of 86% indicating that BsG1-huCD3-FEALxCD30-MDX060-FERR was still able to induce potent T-cell-mediated cytotoxicity in vitro in the presence of sCD30.

Thus, sCD30 concentrations varied between cell types, and correlated with the level of CD30 expression on the cell surface. Furthermore, BsG1-huCD3-FEALxCD30-MDX060-FERR was still able to induce potent T-cell mediated cytotoxicity in vitro in the presence of sCD30.

Example 16 – *Ex vivo* cytotoxicity of bsG1-huCD3-FEALxCD30-MDX060-FERR using patient-derived peripheral blood mononuclear T cells as effector cells

CD3xCD30 bispecific antibodies were tested in an *ex vivo* cytotoxicity assay using a CD30-positive tumor cell line as target cells and primary patient-derived T cells as effector cells. As a source of T cells, Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL) and acute myeloid leukemia (AML) patient-derived peripheral blood mononuclear cells (PBMCs, Discovery Life Sciences, Table 11) were used to evaluate CD3-dependent tumor cell kill.

L-428 tumor cells were labeled with Celltrace FarRed (Invitrogen, cat. no. C34564A; final concentration 2 µM) for 15 min at 37°C. After labeling, 5× the volume of ice cold DBSI was and incubated for 5 minutes at RT. Cells were pelleted, resuspended in medium, and tumor cells were seeded into 96-well plates (Greiner-bio-one, The Netherlands, cat. no. 655180) at a density of 50,000 cells/well. Serial dilutions of bsG1-huCD3-FEALxCD30-MDX060-FERR and control antibody IgG1-b12-FEAL were added (final concentration ranging from 1,000 to 0.051 ng/mL; 3-fold dilutions) and plates were incubated for 15 min at RT. PBMCs were thawed, counted and resuspended in medium (RPMI1640/10% FBS/1% penicillin-streptomycin/1% glutamate), before adding to tumor cells at an effector to target (E:T) ratio of 8:1 and plates were incubated for 72 hours at 37°C. After washing 2 times with PBS/0.1% BSA/0.02% azide (staining buffer), cells were stained to distinguish different cell populations using CD3 (T cells; Invitrogen, cat. no. 48-0037), CD14 (monocytes/macrophages; Biolegend; cat. no. 301834), CD19 (B cells; Biolegend; cat. no. 302246), CD16 (monocytes/macrophages; BD Biosciences; cat. no. 556618), CD56 (NK cells; BD Biosciences; cat. no. 564849) and CD66b (granulocytes; Biolegend; cat. no. 305116). Cells were additionally stained for T cell markers CD4 (1:50; Biolegend, cat. no. 300521, conjugated to Pacific Blue), CD8 (1:100; BD Biosciences, cat. no. 345772, conjugated to FITC) and T cell activation markers CD69 (1:50; Biolegend, cat. no. 310934, conjugated to BV650), CD25 (1:100; Invitrogen, cat. no. 25-0259-42, conjugated to PE-Cy7) and CD279/PD1 (1:50; Biolegend, cat. no. 329930, 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 twice with staining buffer, and cells were stained with 7-AAD (diluted 1:100 in staining buffer) for 10 min at 4°C. Cells were analyzed using a FACS Celesta (BD Biosciences) and data were processed using FlowJo (BD Biosciences). Dose-response curves were generated using non-linear regression analysis

(sigmoidal dose-response with variable slope) using GraphPad Prism V7.02 software (GraphPad Software, San Diego, CA, USA).

The percentage of live target cells was calculated using the following equation:-
% live target cells = (absolute number of live, single Celltrace FarRed-labeled cells in each condition/ absolute number of live, single Celltrace FarRed labeled cells in the condition containing only target cells and T cells without adding any antibodies) x 100.

Results

Figure 25A shows that bsG1-huCD3-FEALxCD30-MDX060-FERR induced dose-dependent cytotoxicity of L-428 tumor cells after 72 h, mediated by T cells derived from both a healthy control donor and different HL and NHL patient donors. Cytotoxicity was associated with T-cell activation and proliferation, illustrated by upregulation of CD69, CD25 and PD-1 **(Figure 25 B-D)**. No T-cell mediated cytotoxicity was observed for control antibody IgG1-b12-FEAL. No T-cell mediated cytotoxicity of L-428 tumor cells was observed for donor E (AML), which could be attributed to the low frequency of T cells within the PBMC sample **(Table 11)**.

Together, these data illustrate that peripheral blood T cells from HL and NHL patients are capable of inducing T-cell mediated cytotoxicity of tumor cell lines in the presence of bsG1-huCD3-FEALxCD30-MDX060-FERR.

Table 11. Primary patient PBMC sample characteristics.

Sample #	**Code**	**Origin**	**Treatment (Tx) status**	**Company**	**Cell viability (%)**	**Frequency of T cells (%)**
A	100000537	Hodgkin, Nodular Sclerosis Hodgkin Lymphoma	stage IV, pre-Tx	Discovery Life Sciences	63.5	71.1
B	120328910	Hodgkin lymphoma	pre-Tx		81.7	56.1
C	17A0D69A3	Hodgkin, Mixed Cellularity Hodgkin Lymphoma	pre-Tx		87.5	35.6

D	D4931CBAB	Hodgkin, Nodular Lymphocyte Predominance Hodgkin Lymphoma	stage II-A, pre-Tx, CD20+		63.95	59.9
E	200003963	Acute Myeloid Leukemia (AML)	active-Tx, EBV tested		65.55	17.4
F	121426858	NHL, Peripheral T-Cell Lymphoma (PTCL) – Unspecified	stage III, post_Tx, treatment CHP+Adcetris/Adriamycin		66.2	68
HC		Healthy control PBMCs	N.A.	Sanquin	86.55	48.4

Example 17 - Evaluation of pharmacokinetic properties of BsG1-huCD3-FEALxCD30-MDX060-FERR in SCID mice

11-12 weeks old, female tumor-free SCID mice (C.B-17/IcrHan®Hsd-Prkdcscid mice, Envigo) (3 mice per group) were injected intravenously (IV) injected with a single dose of 1 µg (0.05 mg/kg) 10 µg (0.5 mg/kg) or 100 µg (5 mg/kg) of BsG1-huCD3-FEALxCD30-MDX060-FERR. The experiment was set up to study antibody clearance in absence of target-mediated clearance, as BsG1-huCD3-FEALxCD30-MDX060-FERR does not cross-react with mouse proteins.

40 µL blood samples were collected from the via cheek vena puncture or vena saphena puncture at 10 minutes, 4-6 hours, 24 hours, 2 days, 7 days, 14 days and 21 days after antibody administration. Blood was collected into K2-EDTA containing vials (Sarstedt, Microvette CB300, cat. No. 16.444.100) and centrifuged for 10 minutes at 10,000 g. Plasma supernatants were transferred to labeled Eppendorf vials and stored at -80°C until plasma IgG concentration determination.

Human IgG concentrations were determined using a total human IgG enzyme-linked immunosorbent assay (ELISA). Mouse anti-human IgG-kappa clone MH16 (CLB Sanquin, The Netherlands; cat. no. M1268), coated in 100 µL PBS (BioTrading, cat. no. K654F500PP) overnight at 4°C to 96-well Microlon ELISA plates (Greiner, Germany) at a concentration of 2 µg/mL, was used as capturing antibody. After blocking plates with PBSA (PBS with 0.2% bovine serum albumin [BSA]) for 1 hour at room temperature (RT), samples were added, serially diluted in PBSA, and incubated on a plate shaker for 1 hour at RT. Plates were washed three times with 300 µL PBST (PBS supplemented with 0.05% Tween 20) and subsequently incubated for 1 hour at RT with goat anti-human IgG immunoglobulin (Jackson, West Grace, PA; cat. no. 109-035-098; 1:10.000 in PBST supplemented with 0.2% BSA). Plates were washed three times with 300 µL PBST before incubation with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, cat. no. 11112422001 and 11112597001) protected from light. The reaction was stopped by adding 100 µL 2% oxalic acid (Sigma-Aldrich, cat. no. 33506), incubated for 10 min at RT. Absorbance was measured at 405 nm on an ELx808 Absorbance microplate reader (Biotek, Winooski, VT).

From the reference antibody human IgG1λ (pure protein 30C, cat. No. BP078) a standard curve was generated (concentration range: 1 mg/mL [3 µL]) to be further diluted in 3-fold dilutions in PBSTA. The injected material was used to generate a second standard curve and was prepared from the 5 mg/kg dose with a concentration of 1 mg/mL (3.6 µL of antibody) to be further diluted in 3-fold dilutions in PBSTA.

Results

From the reference standards a calibration curve was calculated by interpolation of unknowns using a 4-parameter logistic fit curve in Microsoft Excel. Human IgG1 concentrations in the plasma samples were calculated from the equation of the calibration curve, plotted (**Figure 26A**), and the area under the curve (AUC) was calculated using GraphPad Prism software. IgG clearance until the last day of blood sampling (day 21) was determined by the formula D*1.000/AUC, in which D is the dose of injection (1 mg/kg) (**Figure 26B**).

BsG1-huCD3-FEALxCD30-MDX060-FERR does not cross-react with mouse proteins and pharmacokinetic properties would thus be expected to be comparable to other non-binding wild-type human IgG1 molecules. The expected human IgG plasma concentration for all dose groups was calculated to be approximately 100 µg/mL (~5 mg/kg), 10 µg/mL (~0.5 mg/kg) or 1 µg/mL (~0.05 mg/kg). For all time points, samples from animals treated with 0.05 mg/kg were not measurable.

The plasma clearance rate of BsG1-huCD3-FEALxCD30-MDX060-FERR was comparable to the predicted plasma clearance rate of regular human IgG1. The mean maximum human IgG plasma concentration (Cmax) was comparable to the predicted Cmax of regular human IgG1.

Thus, the pharmacokinetic profile of BsG1-huCD3-FEALxCD30-MDX060-FERR is comparable with that predicted for regular human IgG1 in non-tumor bearing SCID mice in absence of target binding.

Example 18- Evaluation of C1q binding to BsG1-huCD3-FEALxCD30-MDX060-FERR

The binding of complement protein C1q to membrane-bound BsG1-huCD3-FEALxCD30-MDX060-FERR, or the parental antibodies from which BsG1-huCD3-FEALxCD30-MDX060-FERR was generated, i.e., IgG1-huCD3-FEAL and IgG1-CD30-MDX060-FERR, was assessed using either CD3- or CD30-expressing cells.-

A. *C1q binding to BsG1-huCD3-FEALxCD30-MDX060-FERR bound to CD3–expressing cells*

Binding of complement protein C1q to CD3-bound BsG1-huCD3-FEALxCD30-MDX060-FERR and IgG1-huCD3-FEAL was tested using stimulated human $CD8^+$ T-cells. IgG1-CD52-E430G was included as a positive control, which has VH and VL domains based on the CD52 antibody CAMPATH-1H and has an Fc-enhanced backbone that is known to efficiently bind C1q when bound to the cell surface. As non-binding negative control antibodies, IgG1-b12-FERR and IgG1-b12 were included.

Human $CD8^+$ T cells were purified (enriched) from buffy coats obtained from healthy volunteers (Sanquin) by negative selection using the RosetteSep™ Human $CD8^+$ T Cell Enrichment Cocktail (Stemcell Technologies, cat. No. 15023C.2) according to the manufacturer's instructions. Purified T cells were resuspended in T-cell medium (Roswell Park Memorial Institute [RPMI]-1640 medium with 25 mM HEPES and L-glutamine (Lonza, cat. No. BE12-115F), supplemented with 10% heat-inactivated donor bovine serum with iron (DBSI; Gibco, cat. No. 20731-030) and penicillin/streptomycin (pen/strep; Lonza, cat. No. DE17-603E).

Anti-CD3/CD28 beads (Dynabeads™ Human T-Activator CD3/CD28; ThermoFisher Scientific, cat. No. 11132D) were washed with PBS and resuspended in T-cell medium. The beads were added to the enriched human $CD8^+$ T cells at a 1:1 ratio and incubated at 37°C, 5% CO_2 for 48 h. Next, the beads were removed using a magnet, and the cells were washed twice in PBS and counted again.

Binding of BsG1-huCD3-FEALxCD30-MDX060-FERR and IgG1-huCD3-FEAL to the activated $CD8^+$ T cells was confirmed by flow cytometry, using BsG1-huCD3-FEALxCD30-MDX060-FERR and IgG1-huCD3-FEAL (30 µg/mL), and R-phycoerythrin (PE)-conjugated goat-

anti-human IgG $F(ab')_2$ (diluted 1:200 in GMB FACS buffer; Jackson ImmunoResearch, cat. no. 109-116-098).

Activated $CD8^+$ T cells were seeded in a round-bottom 96-well plate (30,000 cells/well), pelleted, and resuspended in 30 µL assay medium (RPMI-1640 with 25 mM HEPES and L-glutamine, supplemented with 0.1% [w/v] bovine serum albumin fraction V (BSA; Roche, cat. no. 10735086001) and penicillin/streptomycin). Subsequently, 50 µL of BsG1-huCD3-FEALxCD30-MDX060-FERR, IgG1-huCD3-FEAL, IgG1-b12-FERR, IgG1-CD52-E430G, or IgG1-b12 (final concentrations of 1.7×10^{-4} – 30 µg/mL in 3-fold dilution steps in assay medium) was added to each of the wells and incubated at 37°C for 15 min to allow the antibodies to bind to the cells.

Human serum (20 µL/well; Sanquin, lot 20L15-02), as a source of C1q, was added to a final concentration of 20%. Cells were incubated on ice for 45 min, followed by two washes with cold GMB FACS buffer and incubation with 50 µL fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C1q (final concentration of 20 µg/mL (DAKO, cat no. F0254); diluted 1:75 in GMB FACS buffer) in the presence or absence of allophycocyanin-conjugated mouse-anti-CD8 (BD Biosciences, cat. no. 555369; diluted 1:50 in GMB FACS buffer) in the dark at 4°C for 30 min. Cells were washed twice with cold GMB FACS buffer, resuspended in 20 µL of GMB FACS buffer supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, cat. no. 03690) and 4′,6-diamidino-2-phenylindole (DAPI) viability dye (1:5,000; BD Pharmingen, cat. no. 564907). C1q binding to viable cells (as identified by DAPI exclusion) was analyzed by flow cytometry on an iQue3 Screener (Intellicyt Corporation). Data were analyzed using iQue software (Intellicyt Corporation, ForeCyt® Enterprise Client Edition 6.2 [R3], Version 6.2.652). Binding curves were analyzed using non-linear regression analysis (sigmoidal dose-response with variable slope) using GraphPad Prism software.

Results

Figure 27A shows that whereas dose-dependent C1q binding was observed to membrane-bound IgG1-CD52-E430G, no C1q binding was observed to membrane-bound BsG1-huCD3-FEALxCD30-MDX060-FERR or IgG1-huCD3-FEAL, or to the non-binding control antibodies.

B. *C1q binding to BsG1-huCD3-FEALxCD30-MDX060-FERR bound to CD30–expressing cells*

Binding of complement protein C1q to CD30-bound BsG1-huCD3-FEALxCD30-MDX060-FERR and IgG1-huCD30-MDX060-FERR was tested using NCEB-1 mantle cell lymphoma cells. As a positive control, IgG1-7D8-E430G (anti-CD20) was included, which does not contain the inertness

mutations and retains the capacity of binding to C1q. As non-binding negative control antibodies, IgG1-b12-FERR was included.

NCEB-1 cells were suspended at a concentration of 2×106 cells/mL in assay medium (RPMI-1640 (Lonza, Switzerland, cat. no. BE12-115F) containing 0.1% bovine serum albumin (BSA, fraction V, Roche, cat, no. 10735086001) and 1% penicillin/streptomycin (Gibco, cat. no. 15140-122). Tumor cells (100,000 cells in 50 μL) were added to 96-wells round bottom plates (Greiner Bio, cat no. 650180,). Next, 30 μL of BsG1-huCD3-FEALxCD30-MDX060-FERR, IgG1-CD30-MDX060-FERR, or control antibodies (final concentrations of 5.6 x10-5 – 10 μg/mL in 3-fold dilution steps diluted in assay medium) is added to each of the wells and incubated at 37°C for 15 min to allow the antibodies to bind to the cells.-

Half of the cells (40 μL of cell suspension, containing antibodies) were plated in a different plate for checking the antibody binding. Binding of BsG1-huCD3-FEALxCD30-MDX060-FERR and IgG1-CD30-MDX060-FERR to NCEB-1 cells was confirmed by flow cytometry, using R-phycoerythrin (PE)-conjugated goat-anti-human IgG $F(ab')_2$ (diluted 1:500 in FACS buffer; Jackson ImmunoResearch, cat. no. 109-116-098).-

Human serum (10 μL/well; Sanquin, lot 21K04-01) was added to the remaining 40μL of cell suspension (20% of final concentration) and incubated on ice for 45 min, followed by washing with FACS buffer and incubation with 25 μL FITC-conjugated rabbit anti-C1q antibodies (final concentration of 20 μg/mL (DAKO, cat no. F0254); diluted in FACS buffer) in the dark at 4°C for 30 min. Cells were washed with cold FACS buffer, resuspended in 30 μL of FACS buffer –supplemented with TO-PRO™-3 viability stain (1:5000; ThermoFisher, cat. no. T3605) and measured on the iQue3 Screener (Intellicyt Corporation). Data were analyzed using iQue software (Intellicyt Corporation, ForeCyt® Enterprise Client Edition 6.2 [R3], Version 6.2.652). Binding curves were analyzed using non-linear regression analysis (sigmoidal dose-response with variable slope) using GraphPad Prism software.

Results

Figure 27B shows that while dose-dependent C1q binding was observed to membrane-bound IgG1-CD20-E430G, no C1q binding was observed to membrane-bound BsG1-huCD3-FEALxCD30-MDX060-FERR or IgG1-CD30-MDX060-FERR, or to the non-binding control antibodies.

Thus, these results demonstrated that bsG1-huCD3-FEALxCD30-MDX060-FERR does not bind C1q, confirming the functionally inert backbone.

CLAIMS

1. A multispecific antibody comprising:

   (i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

   (ii) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively.

2. The multispecific antibody according to claim 1, wherein the first heavy chain variable region and/or the first light chain variable region is human.

3. The multispecific antibody according to claim 1 or claim 2, wherein the second heavy chain variable region and/or the second light chain variable region is humanized.

4. The multispecific antibody according to any one of the preceding claims, wherein X in SEQ ID NO:9 is an H.

5. The multispecific antibody according to any one of claims 1 to 3, wherein X in SEQ ID NO:9 is a G.

6. The multispecific antibody according to any one of the preceding claims, wherein the first heavy chain variable region comprises the sequence set forth in SEQ ID NO:13 and the first light chain variable region comprises the sequence set forth in SEQ ID NO:14.

7. The multispecific antibody according to any one of the preceding claims, wherein the second heavy chain variable region comprises the sequence set forth in SEQ ID NO:15 and the second light chain variable region comprises the sequence set forth in SEQ ID NO:16.

8. The multispecific antibody according to any one of the preceding claims, wherein the multispecific antibody is a bispecific antibody.

9. The multispecific antibody according to any one of the preceding claims, wherein the multispecific antibody comprises an Fc region consisting of a first and second Fc polypeptide.

10. The multispecific antibody according to claim 9, wherein the Fc region is an IgG1 Fc region, preferably a human IgG1 Fc region.

11. The multispecific antibody according to claim 9 or 10, wherein the multispecific antibody is a full-length antibody.

12. The multispecific antibody according to any one of claims 9 to 11, comprising an inert Fc region.

13. The multispecific antibody according to any one of claims 9 to 12, wherein the first and/or second Fc polypeptide comprise(s) a substitution of an amino acid corresponding to the amino acid at positions L234 and/or L235 in a human IgG1 heavy chain, wherein the substitutions preferably are to F and E, respectively, wherein the amino acid positions are as defined by Eu numbering.

14. The multispecific antibody according to claim 12 or 13, wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and/or second Fc polypeptide further comprises a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R, wherein the amino acid positions are as defined by Eu numbering.

15. The multispecific antibody according to claim 14, wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and second Fc polypeptide further comprise a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R.

16. The multispecific antibody according to claim 12 or 13, wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and/or second Fc polypeptide further comprises a substitution of an amino acid corresponding to the amino

acid at position D265 in a human IgG1 heavy chain, wherein the substitution preferably is to A wherein the amino acid positions are as defined by Eu numbering.

17. The multispecific antibody according to claim 16, wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and second Fc polypeptide further comprises a substitution of an amino acid corresponding to the amino acid at position D265 in a human IgG1 heavy chain, wherein the substitution preferably is to A.

18. The multispecific antibody according to any one of claims 12 to 14, wherein one of the first and second Fc polypeptides comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and G236 to F, E and R, respectively, and the other Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235E and D265 to F, E and A, respectively, wherein the amino acid positions are as defined by Eu numbering.

19. The multispecific antibody according to claim 18, wherein the first Fc polypeptide and the first heavy chain variable region are comprised within the same polypeptide chain and wherein the second Fc polypeptide and the second heavy chain variable region are comprised within the same polypeptide chain.

20. The multispecific antibody according to claim 19, wherein the first Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and G236 to F, E and R, respectively, and the second Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235E and D265 to F, E and A, respectively, wherein the amino acid positions are as defined by Eu numbering.

21. The multispecific antibody according to any one of claims 9 to 20, wherein in the first Fc polypeptide 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 heavy chain has been substituted, and in the second Fc polypeptide 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 heavy chain has been substituted, and wherein said substitutions in the first and second Fc polypeptides are not in the same positions, wherein the amino acid positions are as defined by Eu numbering.

22. The multispecific antibody according to claim 21, wherein the amino acid in the position corresponding to F405 is L in the first Fc polypeptide and wherein the amino acid in the position corresponding to K409 is R in the second Fc polypeptide, or vice versa.

23. The multispecific antibody according to claim 22, wherein the amino acid in the position corresponding to K409 is R in the first Fc polypeptide and wherein the amino acid in the position corresponding to F405 is L in the second Fc polypeptide.

24. The multispecific antibody according to any one of the preceding claims, comprising:

(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively;

wherein the multispecific antibody is a bispecific antibody and comprises an Fc region consisting of a first and second Fc polypeptide;

wherein the first Fc polypeptide and the first heavy chain variable region are comprised within the same polypeptide chain and wherein the second Fc polypeptide and the second heavy chain variable region are comprised within the same polypeptide chain;

wherein the first Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and G236 to F, E and R, respectively, and the second Fc polypeptide comprises substitutions of the amino acids corresponding to the amino acids at positions L234, L235 and D265 to F, E and A, respectively, wherein the amino acid positions are as defined by Eu numbering; and

wherein the amino acid in the position corresponding to K409 is R in the first Fc polypeptide and wherein the amino acid in the position corresponding to F405 is L in the second Fc polypeptide.

25. The multispecific antibody according to any one of the preceding claims, comprising or consisting of the heavy chain sequences set forth in SEQ ID NO:17 and 19 and the light chain sequences set forth in SEQ ID NO:18 and 20.

26. The multispecific antibody according to any one of the preceding claims, comprising or consisting of the heavy chain sequences set forth in SEQ ID NO:17 and 19 and the light chain sequences set forth in SEQ ID NO:18 and 20, wherein the multispecific antibody is a bispecific antibody.

27. The multispecific antibody according to any one of claims 1 to 8, wherein
(i) the CD30 binding region and/or the CD3 binding region is a Fab,
(ii) the CD30 binding region and/or the CD3 binding region is an scFv,
(iii) the CD30 binding region is Fab and the CD3 binding region is an scFv, or
(iv) the CD30 binding region is an scFv and the CD3 binding region is a Fab.

28. A nucleic acid construct, or a combination of nucleic acid constructs, encoding the multispecific antibody according to any one of the preceding claims.

29. An expression vector, or a combination of expression vectors, comprising the nucleic acid construct(s) according to claim 28.

30. A delivery vehicle comprising the nucleic acid construct(s) according to claim 28.

31. The delivery vehicle according to claim 30, wherein said delivery vehicle is a particle.

32. The delivery vehicle according to claim 31, wherein said particle is a lipid nanoparticle.

33. The delivery vehicle according to claim 32, wherein said lipid nanoparticle comprises lipids, ionizable aminolipids, PEG-lipids, cholesterol or any combination thereof.

34. A recombinant host cell capable of producing the multispecific antibody according to any one of claims 1 to 27, wherein the host cell comprises one or more nucleic acid constructs encoding the multispecific antibody according to any one of the preceding claims.

35. The recombinant host cell according to claim 34, wherein the recombinant host cell is a CHO cell.

36. A pharmaceutical composition comprising a multispecific antibody according to any one of claims 1 to 27 and a pharmaceutically-acceptable carrier.

37. The multispecific antibody according to any one of the claims 1 to 27, the nucleic acid constructs according to claim 28, the delivery vehicle according to any one of the claims 30 to 33 or the pharmaceutical composition according to claim 36 for use as a medicament.

38. The multispecific antibody according to any one of the claims 1 to 27, the nucleic acid constructs according to claim 28, the delivery vehicle according to any one of the claims 30 to 33 or the pharmaceutical composition according to claim 36 for use in the treatment of cancer.

39. The multispecific antibody according to any one of the claims 1 to 27, the nucleic acid constructs according to claim 28, the delivery vehicle according to any one of the claims 30 to 33 or the pharmaceutical composition according to claim 36 for use in the treatment of Hodgkin's lymphoma or Non-Hodgkin's lymphoma (NHL).

40. The multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition for use according to claim 39, wherein Non-Hodgkin's lymphoma is T cell Non-Hodgkin's lymphoma (T-NHL).

41. The multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition for use according to claim 40, wherein T-NHL is cutaneous T-cell lymphoma (CTCL) or Peripheral T-cell lymphoma (PTCL).

42. The multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition for use according to any one of the claims 39 to 41, wherein T cell Non-Hodgkin's lymphoma (T-NHL) is anaplastic large cell lymphoma (ALCL).

43. The multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition for use according to claim 39, wherein Non-Hodgkin's lymphoma is B cell Non-Hodgkin's lymphoma (B-NHL).

44. The multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition for use according to any one of the claims 37 to 43, wherein the multispecific antibody, the nucleic acid constructs, the delivery vehicle or the pharmaceutical composition has been administered intravenously and/or subcutaneously, preferably subcutaneously.

45. A method for producing a multispecific antibody according to any one of claims 1 to 27, comprising
(i) culturing the recombinant host cell of claim 34 or 35 under conditions wherein the antibody is produced, and
(ii) isolating the produced multispecific antibody from the culture.

46. A method for producing a multispecific antibody according to any one of claims 9 to 26, comprising
a) providing a first antibody comprising the CD30 binding region as described in claim 1 (i), and a second antibody comprising the CD3 binding region as described in claim 1 (ii), wherein said antibodies optionally contain the further features described in claims 2 to 26,
wherein the first and second antibodies comprise an Fc region, and
wherein the sequences of the first and second CH3 regions of the first and second antibodies are different and are such that the heterodimeric interaction between the first and second CH3 regions is stronger than each of the homodimeric interactions of the first and second CH3 regions;
b) incubating the first antibody together with the second antibody under reducing conditions sufficient to allow the cysteines in the hinge regions to undergo disulfide-bond isomerization; and
c) obtaining the multispecific antibody comprising the first immunoglobulin heavy chain and the first immunoglobulin light chain of the first antibody and the second immunoglobulin heavy chain and the second immunoglobulin light chain of the second antibody.

47. 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 27, comprising an antibody as defined in any one of claims 1 to 27; and instructions for use of said kit.

48. A diagnostic composition comprising a multispecific antibody according to any one of the claims 1 to 27.

49. An antibody comprising
(i) a CD30 binding region comprising a first heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a first light

chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and
(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and second Fc polypeptide further comprise a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R, wherein the amino acid positions are as defined by Eu numbering.

50. The antibody according to claim 49, wherein the first heavy chain variable region comprises the sequence set forth in SEQ ID NO:13 and the first light chain variable region comprises the sequence set forth in SEQ ID NO:14.

51. The antibody according to any one of the claims 49 to 50, wherein the antibody comprises or consists of the heavy chain sequences set forth in SEQ ID NO:17 and the light chain sequences set forth in SEQ ID NO:18.

52. An antibody comprising
(i) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively, and
(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and wherein the first and second Fc polypeptide further comprise a substitution of an amino acid corresponding to the amino acid at position G236 in a human IgG1 heavy chain, wherein the substitution preferably is to R, wherein the amino acid positions are as defined by Eu numbering.

53. The antibody according to claim 52, wherein X in SEQ ID NO:9 is an H.

54. The antibody according to claim 52, wherein X in SEQ ID NO:9 is a G.

55. The antibody according to any one of the claims 52 to 54, wherein the second heavy chain variable region comprises the sequence set forth in SEQ ID NO:15 and the second light chain variable region comprises the sequence set forth in SEQ ID NO:16.

56. The antibody according to any one of claims 49 to 55, wherein the antibody is a full-length antibody.

57. The antibody according to any one of claims 49 to 56, wherein 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 heavy chain has been substituted, wherein preferably, the amino acid in the position corresponding to F405 is L or the amino acid in the position corresponding to K409 is R.

58. A method for producing a multispecific antibody, comprising

a) providing a first antibody as described in any one of the claims 49 to 51 or 55 to 57 and a second antibody comprising

(i) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and a substitution of an amino acid corresponding to the amino acid at position D265 to A in a human IgG1 heavy chain,

or

providing a second antibody as described in any one of the claims 52 to 57 and a first antibody comprising

(i) a CD30 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:1, 2 and 3, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:4, 5 and 6, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and a substitution of an amino acid corresponding to the amino acid at position D265 to A in a human IgG1 heavy chain,

wherein the sequences of the first and second CH3 regions of the first and second antibodies are different and are such that the heterodimeric interaction between the first and second CH3 regions is stronger than each of the homodimeric interactions of the first and second CH3 regions; wherein preferably the amino acid in the position corresponding to F405 is L in the first CH3 region and the amino acid in the position corresponding to K409 is R in the second CH3 region, or vice versa,

b) incubating the first antibody together with the second antibody under reducing conditions sufficient to allow the cysteines in the hinge regions to undergo disulfide-bond isomerization; and

c) obtaining the multispecific antibody comprising the first immunoglobulin heavy chain and the first immunoglobulin light chain of the first antibody and the second immunoglobulin heavy chain and the second immunoglobulin light chain of the second antibody.

59. The method for producing a multispecific antibody according to claim 58, comprising

a) providing a first antibody as described in any one of the claims 49 to 51 or 55 to 57 and a second antibody comprising

(i) a CD3 binding region comprising a second heavy chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:7, 8 and 9, respectively, and a second light chain variable region comprising the CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NO:10, 11 and 12, respectively, and

(ii) an Fc region consisting of a first and second Fc polypeptide wherein the first and second Fc polypeptides comprise substitutions of the amino acids corresponding to the amino acids at positions L234 and L235 to F and E, respectively, and a substitution of an amino acid corresponding to the amino acid at position D265 to A in a human IgG1 heavy chain,

wherein the sequences of the first and second CH3 regions of the first and second antibodies are different and are such that the heterodimeric interaction between the first and second CH3 regions is stronger than each of the homodimeric interactions of the first and second CH3 regions; wherein preferably the amino acid in the position corresponding to K409 is R in the first CH3 region and the amino acid in the position corresponding to F405 is L in the second CH3 region,

b) incubating the first antibody together with the second antibody under reducing conditions sufficient to allow the cysteines in the hinge regions to undergo disulfide-bond isomerization; and

c) obtaining the multispecific antibody comprising the first immunoglobulin heavy chain and the first immunoglobulin light chain of the first antibody and the second immunoglobulin heavy chain and the second immunoglobulin light chain of the second antibody.

60. A multispecific antibody obtained by a method as described in any of the claims 58 to 59.

Figure 1

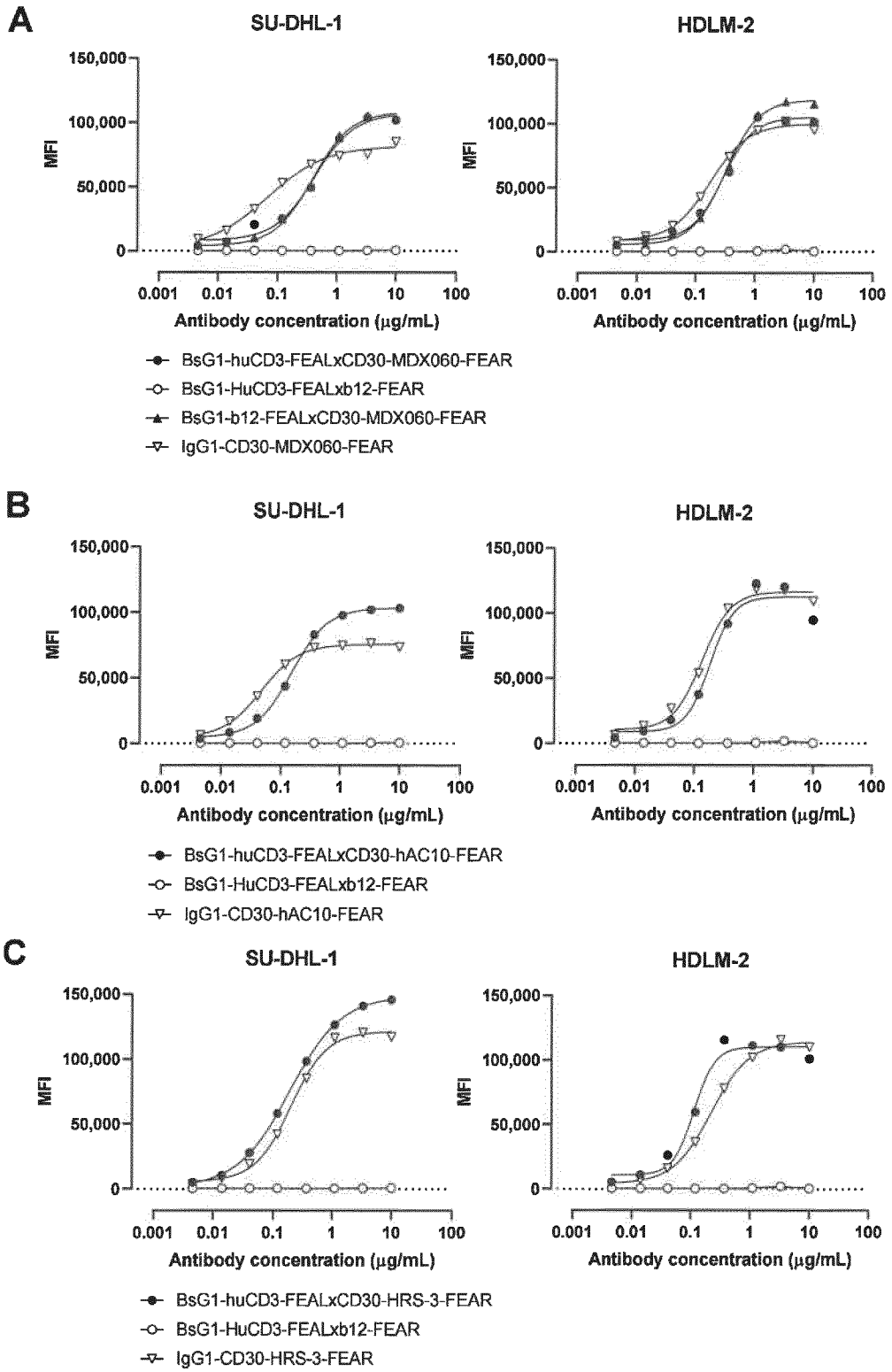

A
SU-DHL-1
HDLM-2
MFI
150,000
100,000
50,000
0
0.001
0.01
0.1
1
10
100
Antibody concentration (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FEAR
BsG1-HuCD3-FEALxb12-FEAR
BsG1-b12-FEALxCD30-MDX060-FEAR
IgG1-CD30-MDX060-FEAR
B
SU-DHL-1
HDLM-2
BsG1-huCD3-FEALxCD30-hAC10-FEAR
BsG1-HuCD3-FEALxb12-FEAR
IgG1-CD30-hAC10-FEAR
C
SU-DHL-1
HDLM-2
BsG1-huCD3-FEALxCD30-HRS-3-FEAR
BsG1-HuCD3-FEALxb12-FEAR
IgG1-CD30-HRS-3-FEAR

Figure 1 (continued)

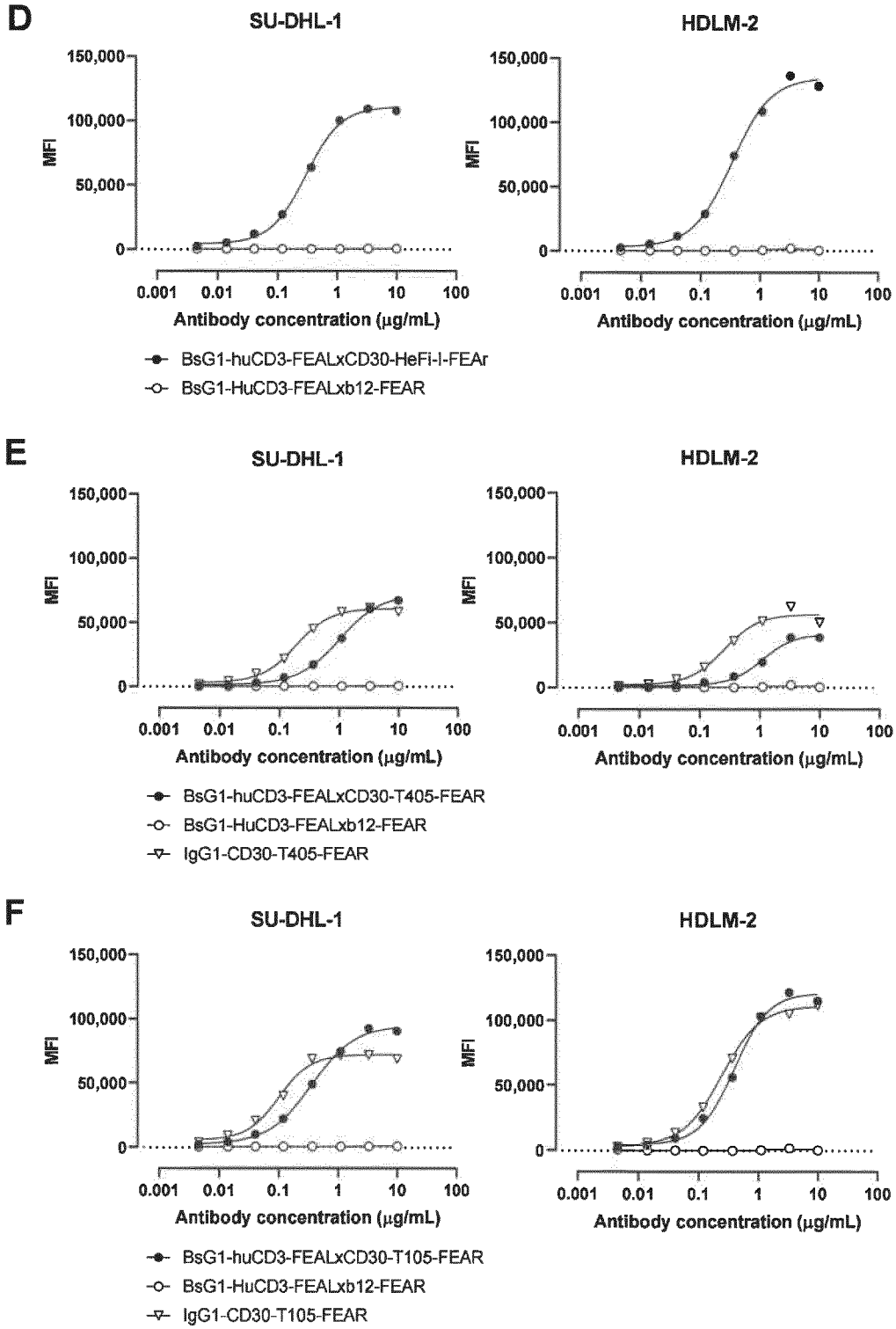
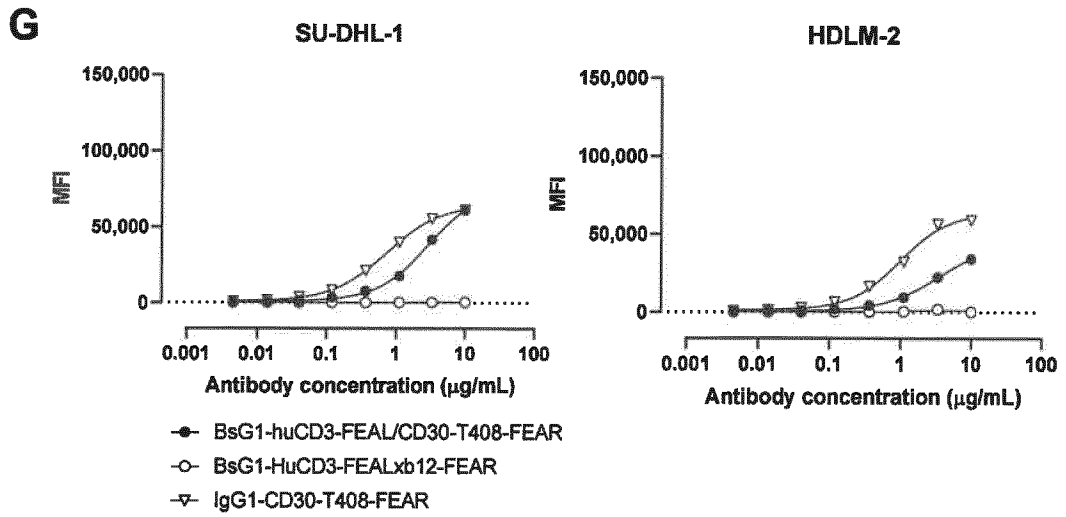
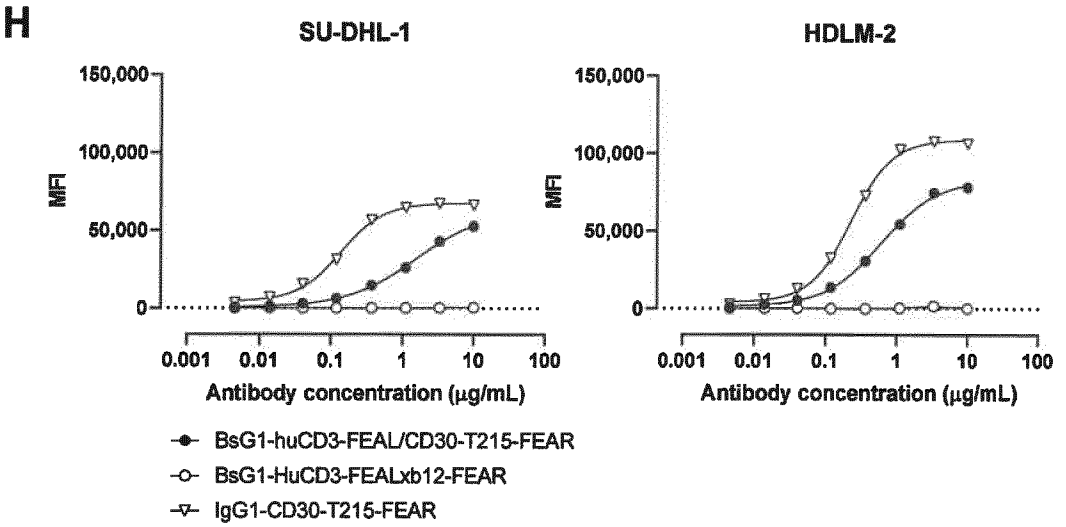


Figure 1 (continued)





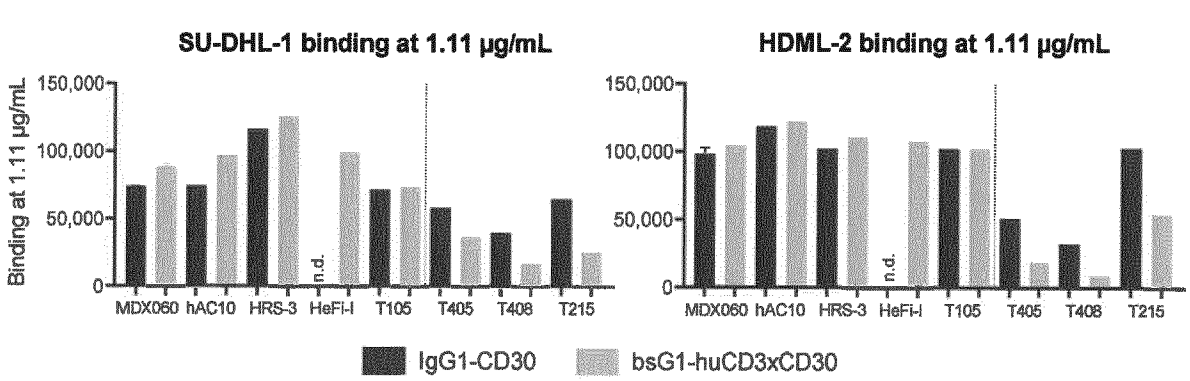


Figure 2

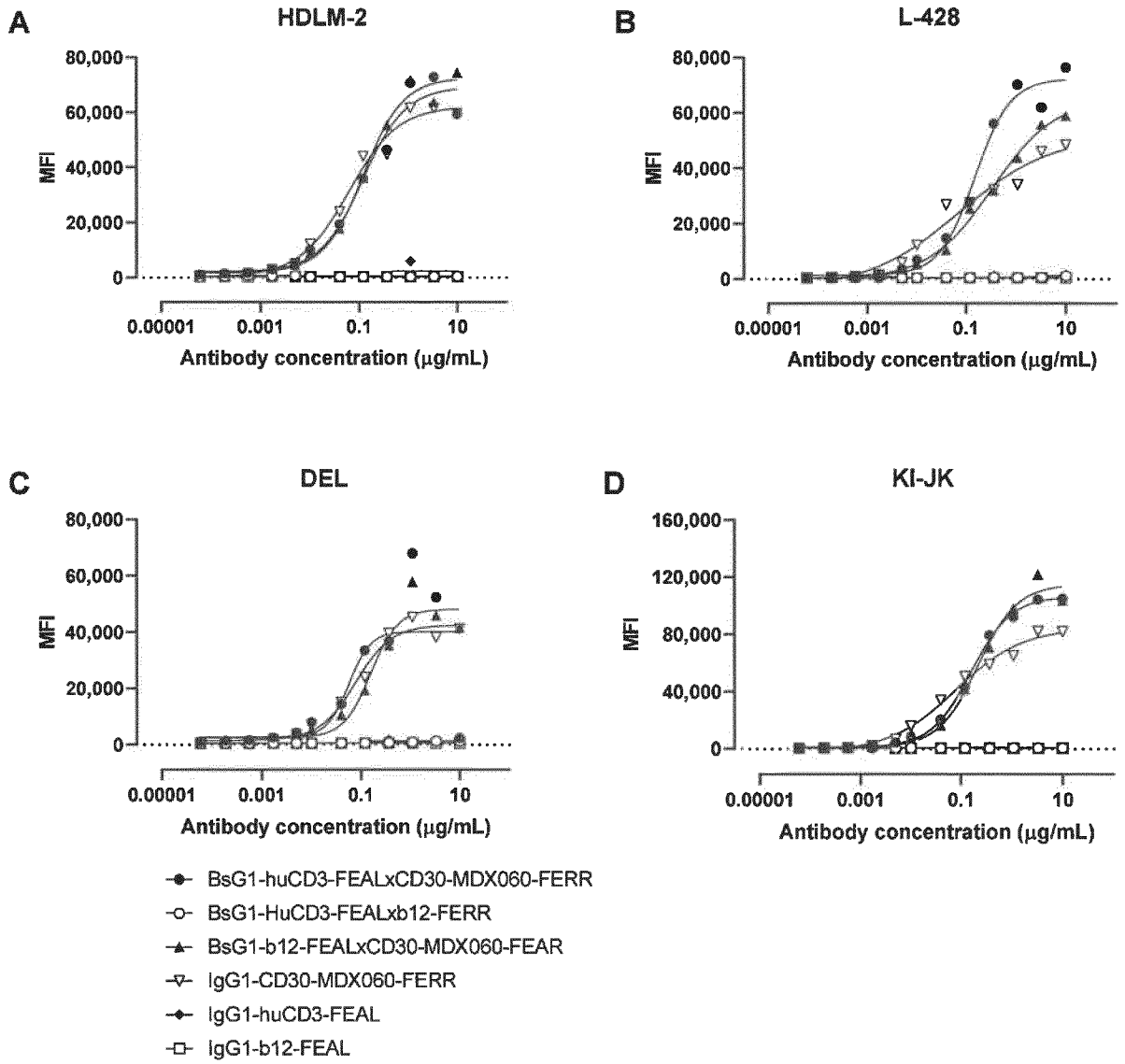

A
HDLM-2
B
L-428
C
DEL
D
KI-JK
MFI
80,000
60,000
40,000
20,000
0
160,000
120,000
0.00001
0.001
0.1
10
Antibody concentration (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FERR
BsG1-HuCD3-FEALxb12-FERR
BsG1-b12-FEALxCD30-MDX060-FEAR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 3

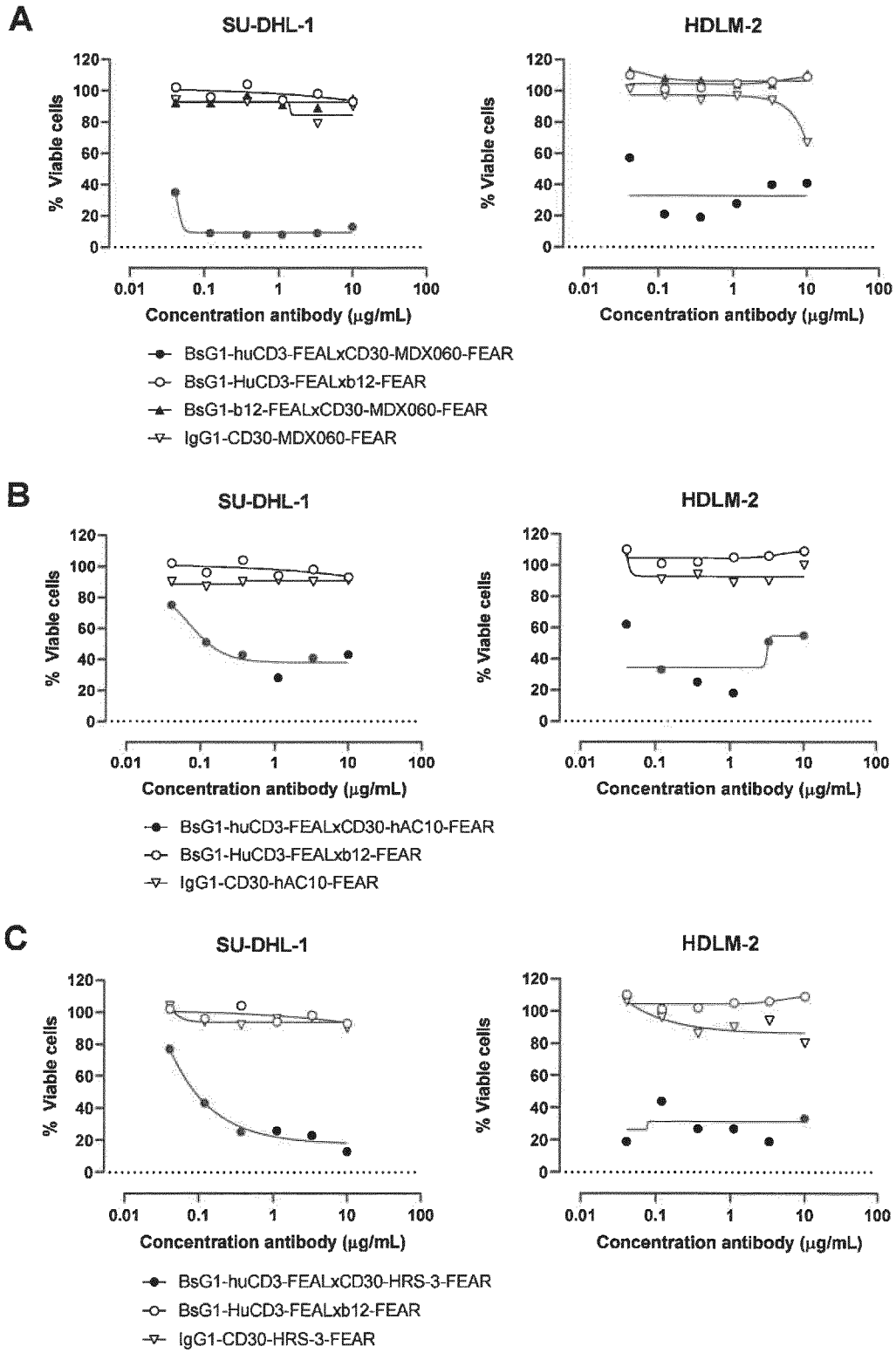

A
SU-DHL-1
HDLM-2
% Viable cells
Concentration antibody (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FEAR
BsG1-HuCD3-FEALxb12-FEAR
BsG1-b12-FEALxCD30-MDX060-FEAR
IgG1-CD30-MDX060-FEAR
B
SU-DHL-1
HDLM-2
% Viable cells
Concentration antibody (µg/mL)
BsG1-huCD3-FEALxCD30-hAC10-FEAR
BsG1-HuCD3-FEALxb12-FEAR
IgG1-CD30-hAC10-FEAR
C
SU-DHL-1
HDLM-2
% Viable cells
Concentration antibody (µg/mL)
BsG1-huCD3-FEALxCD30-HRS-3-FEAR
BsG1-HuCD3-FEALxb12-FEAR
IgG1-CD30-HRS-3-FEAR

Figure 3 (continued)

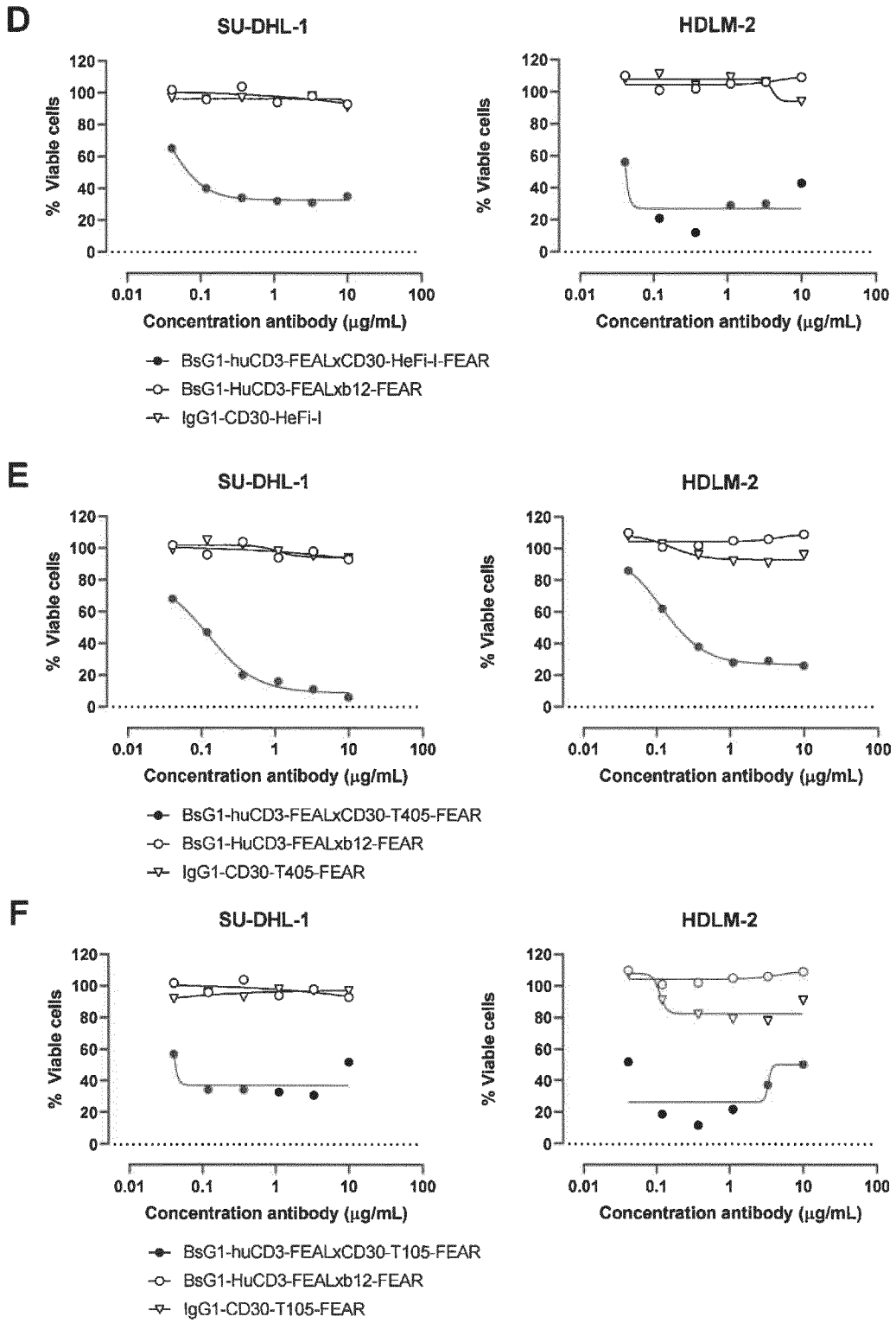
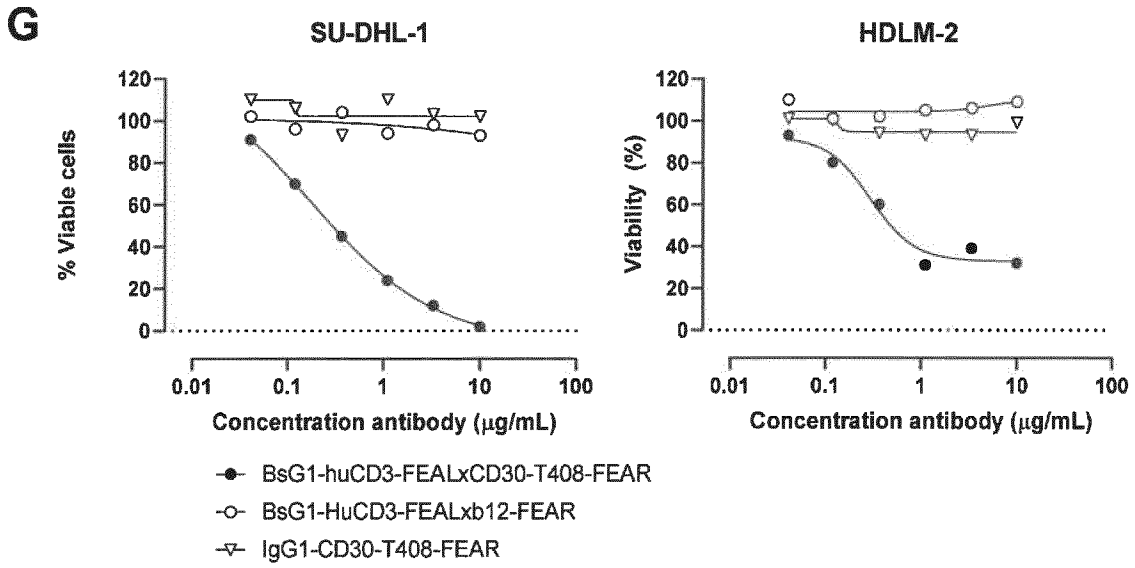


Figure 3 (continued)



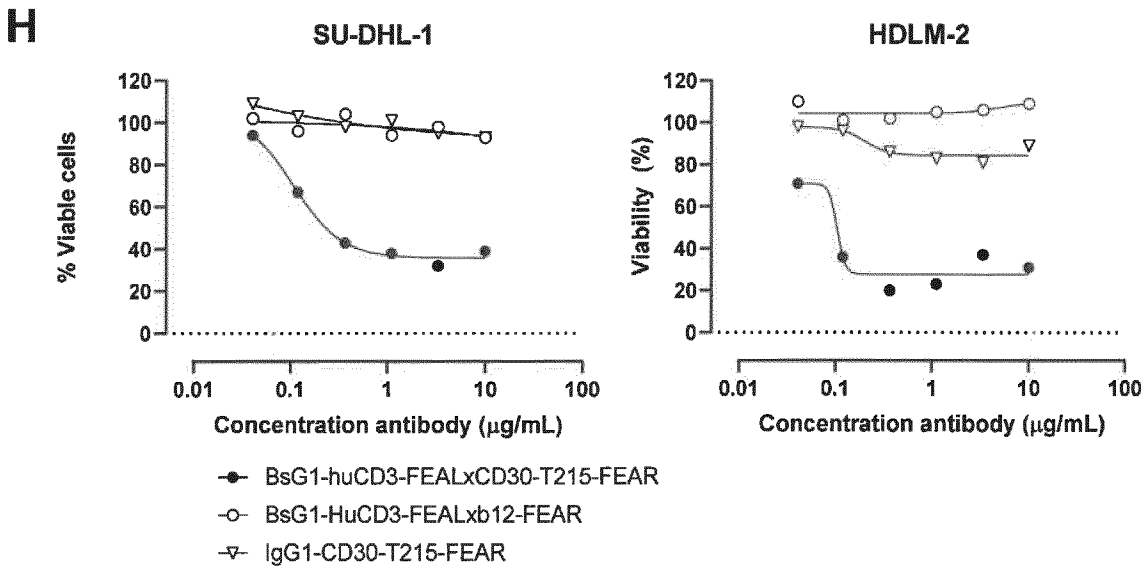


Figure 4

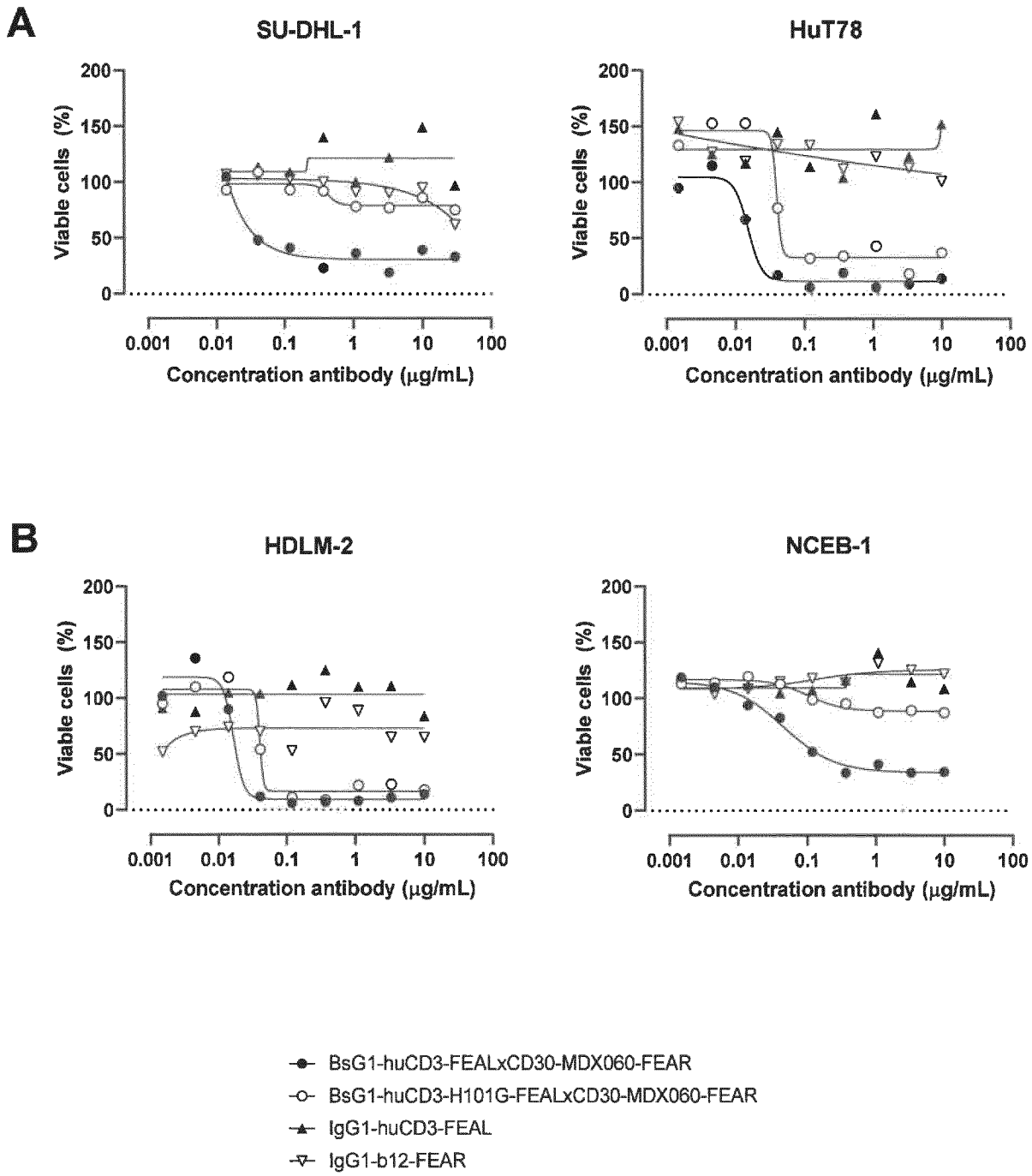
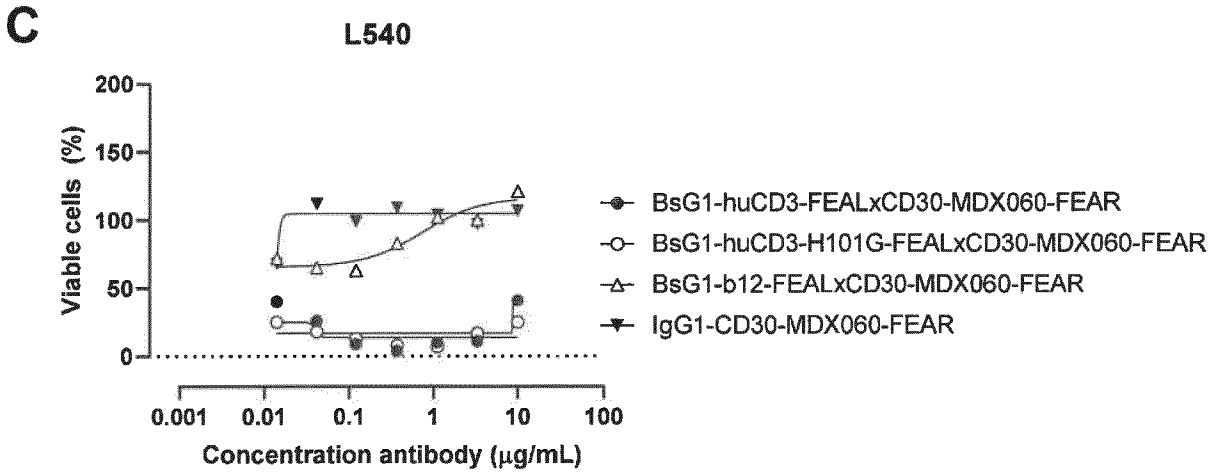

A
SU-DHL-1
HuT78
Viable cells (%)
200
150
100
50
0
0.001
0.01
0.1
1
10
100
Concentration antibody (µg/mL)
B
HDLM-2
NCEB-1
BsG1-huCD3-FEALxCD30-MDX060-FEAR
BsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR
IgG1-huCD3-FEAL
IgG1-b12-FEAR

Figure 4 (continued)



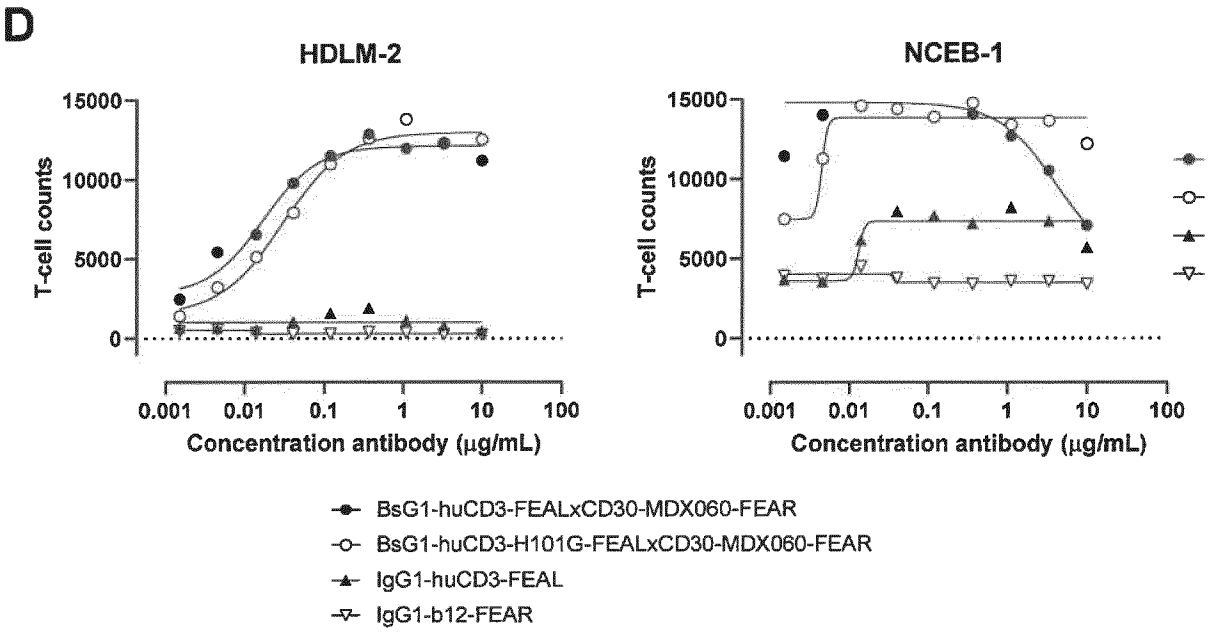


Figure 5

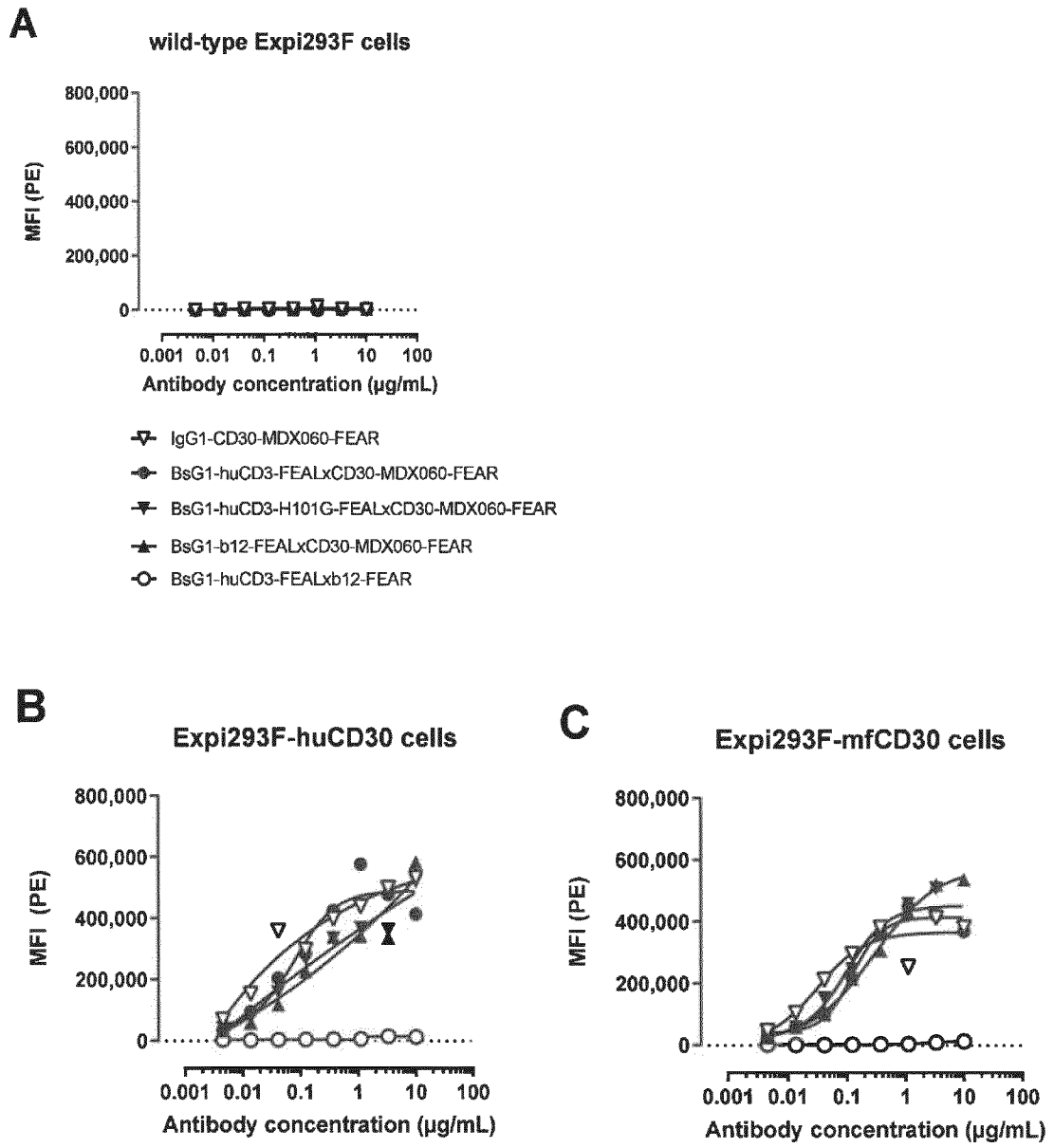

A
wild-type Expi293F cells
MFI (PE)
800,000
600,000
400,000
200,000
0
0.001 0.01 0.1 1 10 100
Antibody concentration (µg/mL)
IgG1-CD30-MDX060-FEAR
BsG1-huCD3-FEALxCD30-MDX060-FEAR
BsG1-huCD3-H101G-FEALxCD30-MDX060-FEAR
BsG1-b12-FEALxCD30-MDX060-FEAR
BsG1-huCD3-FEALxb12-FEAR
B
Expi293F-huCD30 cells
MFI (PE)
800,000
600,000
400,000
200,000
0
0.001 0.01 0.1 1 10 100
Antibody concentration (µg/mL)
C
Expi293F-mfCD30 cells
MFI (PE)
800,000
600,000
400,000
200,000
0
0.001 0.01 0.1 1 10 100
Antibody concentration (µg/mL)

Figure 5 (continued)

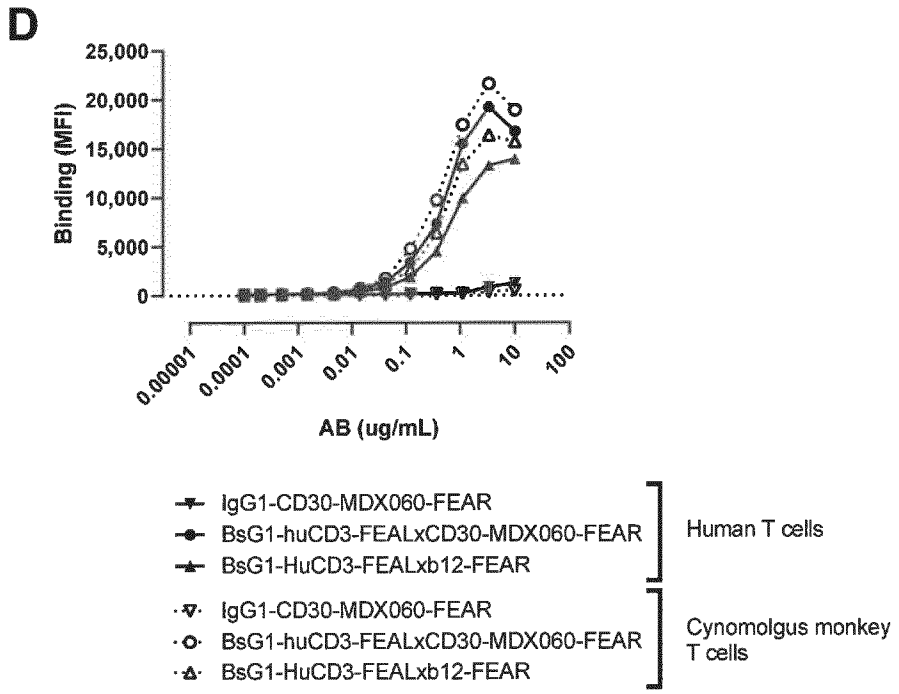
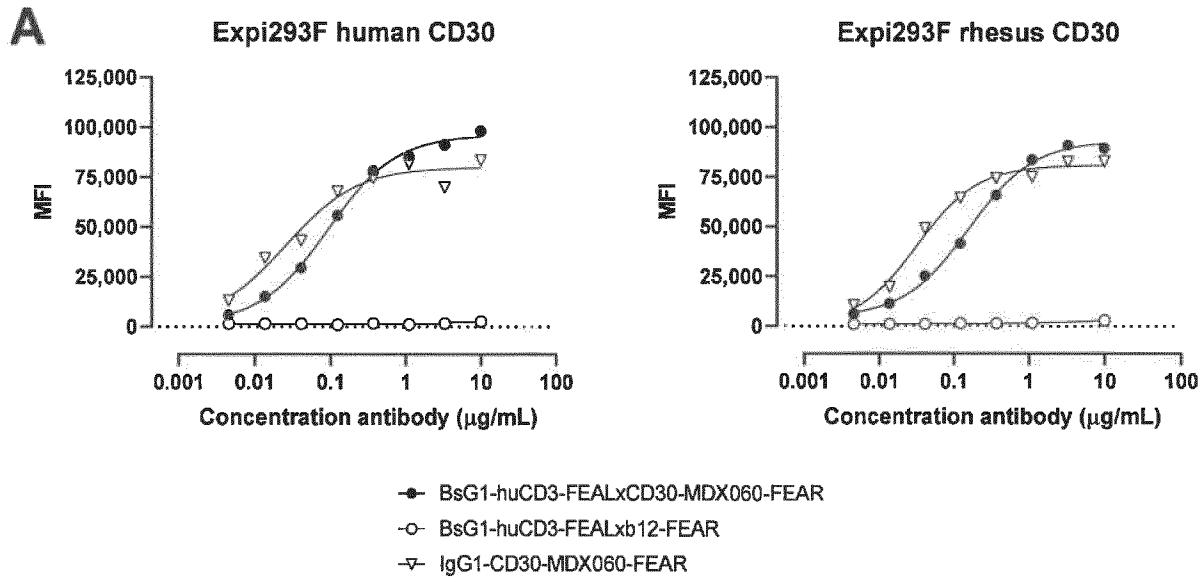


Figure 6


A
Expi293F human CD30
Expi293F rhesus CD30
MFI
125,000
100,000
75,000
50,000
25,000
0
0.001
0.01
0.1
1
10
100
Concentration antibody (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FEAR
BsG1-huCD3-FEALxb12-FEAR
IgG1-CD30-MDX060-FEAR

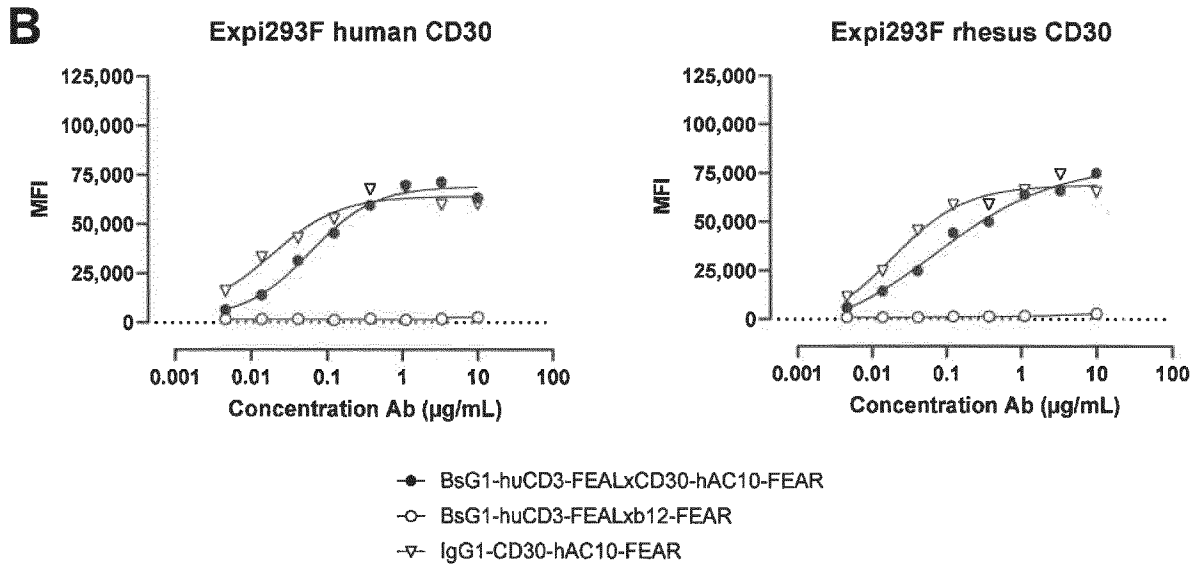
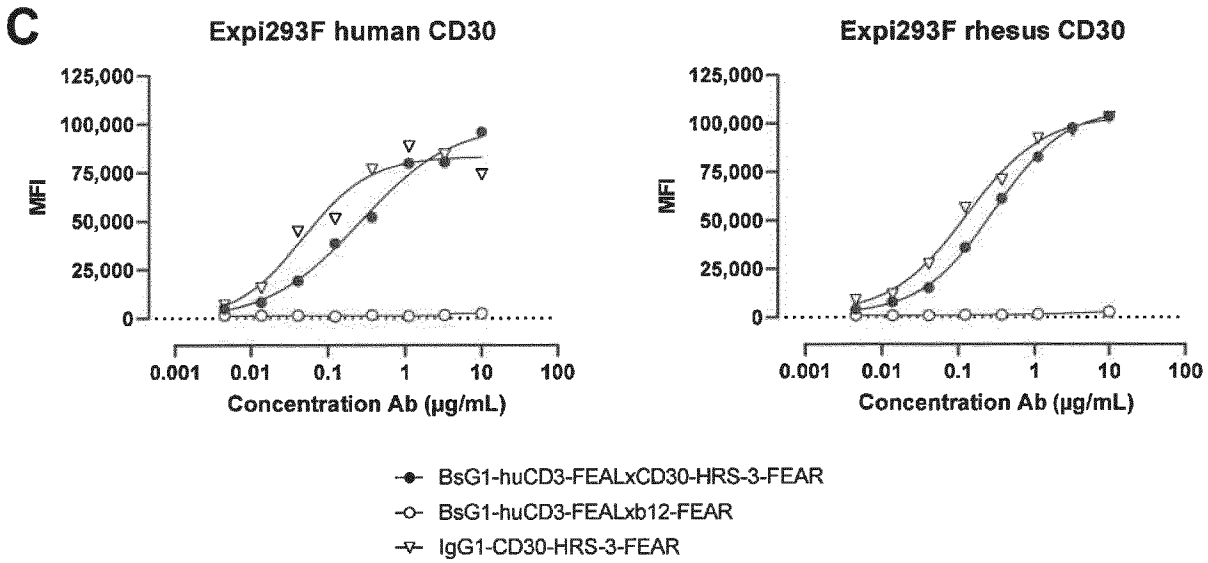

B
Expi293F human CD30
Expi293F rhesus CD30
MFI
125,000
100,000
75,000
50,000
25,000
0
0.001
0.01
0.1
1
10
100
Concentration Ab (µg/mL)
BsG1-huCD3-FEALxCD30-hAC10-FEAR
BsG1-huCD3-FEALxb12-FEAR
IgG1-CD30-hAC10-FEAR

Figure 6 (continued)



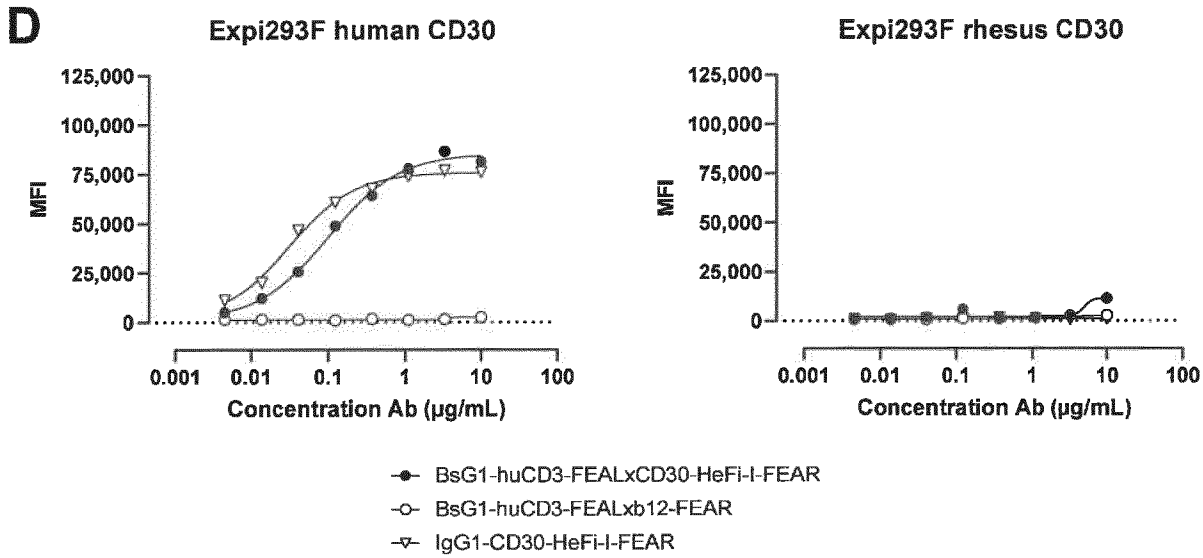
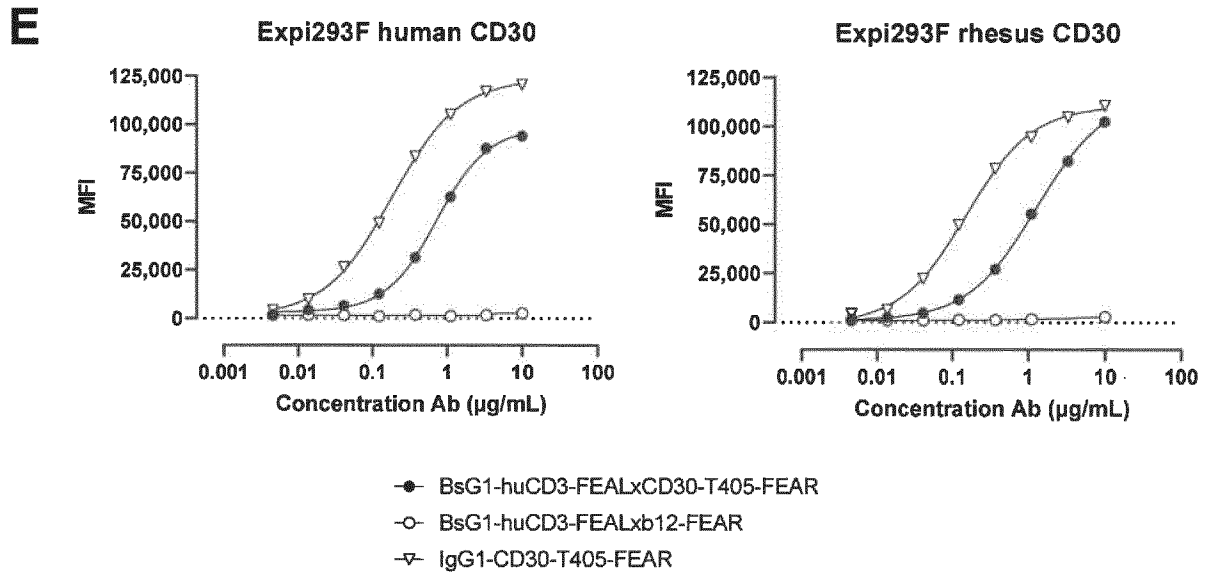


Figure 6 (continued)



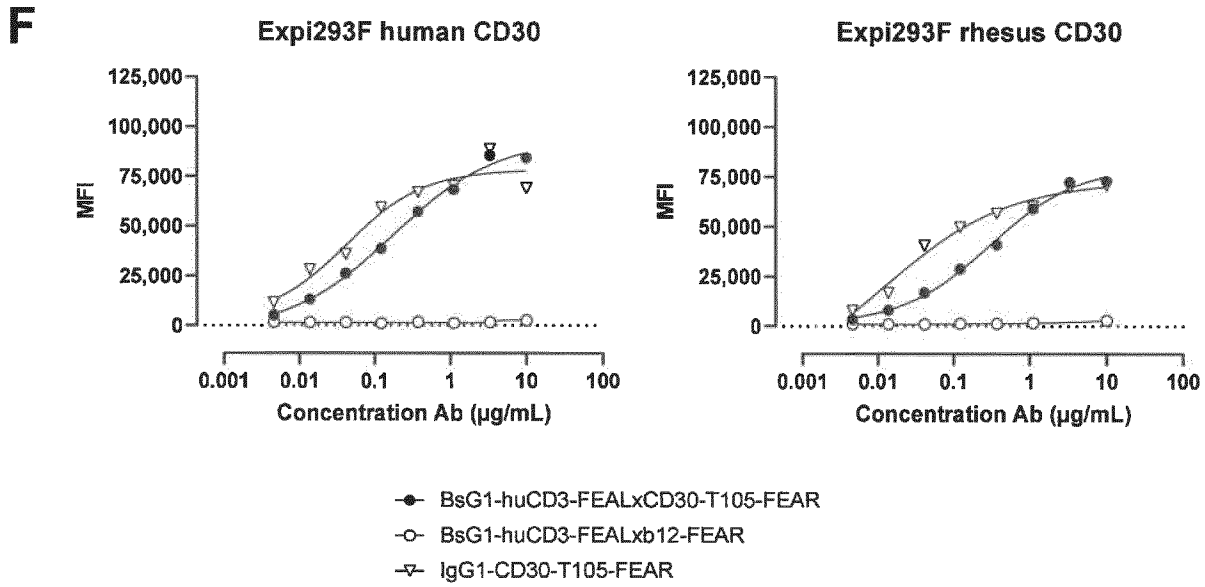
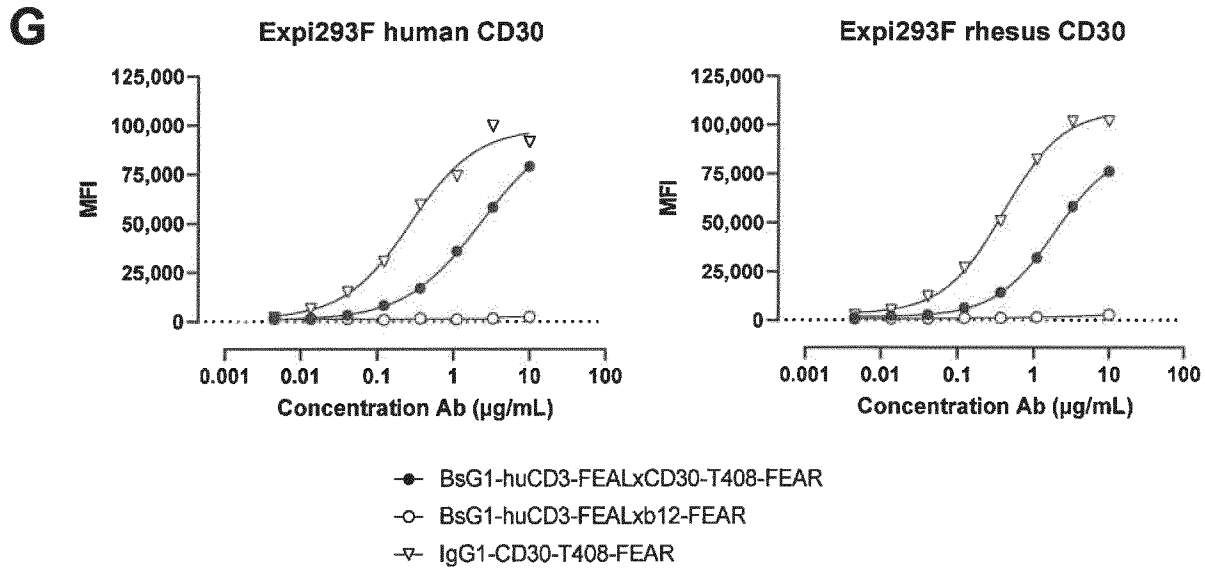


Figure 6 (continued)



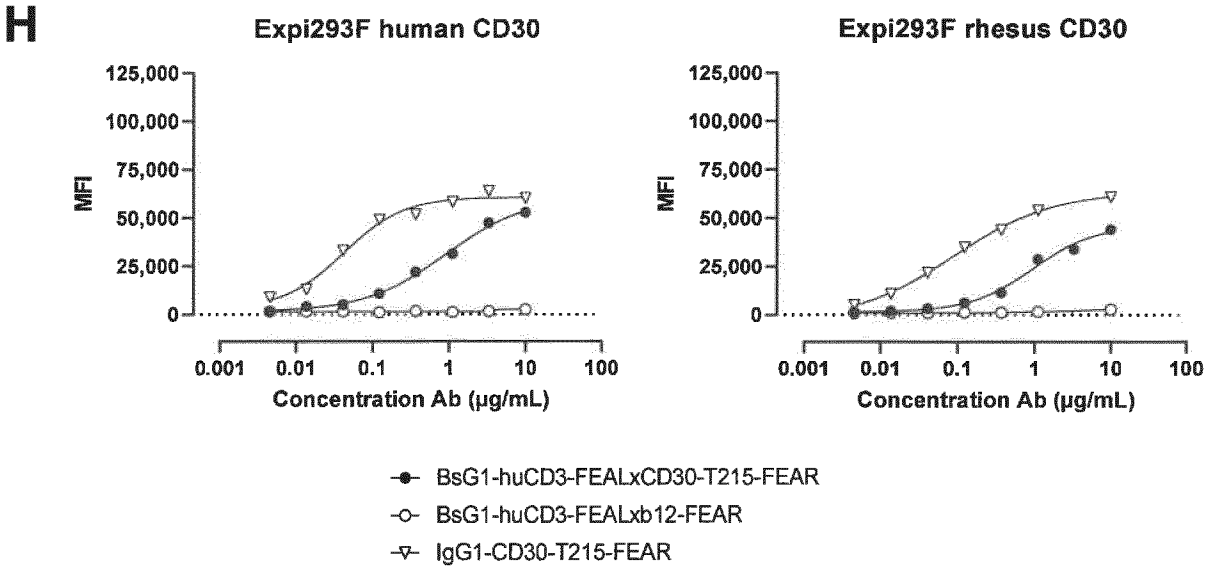


Figure 7

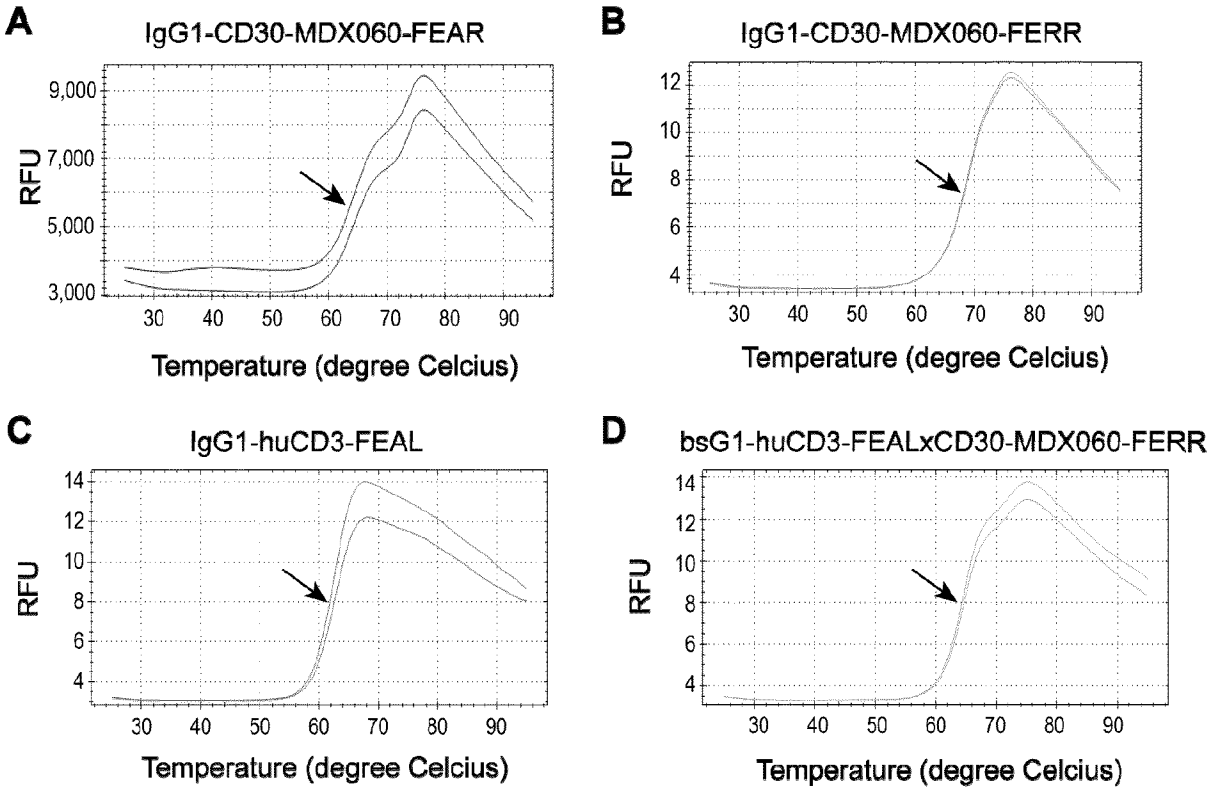

A
IgG1-CD30-MDX060-FEAR
RFU
9,000
7,000
5,000
3,000
30 40 50 60 70 80 90
Temperature (degree Celcius)
B
IgG1-CD30-MDX060-FERR
RFU
12
10
8
6
4
30 40 50 60 70 80 90
Temperature (degree Celcius)
C
IgG1-huCD3-FEAL
RFU
14
12
10
8
6
4
30 40 50 60 70 80 90
Temperature (degree Celcius)
D
bsG1-huCD3-FEALxCD30-MDX060-FERR
RFU
14
12
10
8
6
4
30 40 50 60 70 80 90
Temperature (degree Celcius)

Figure 8

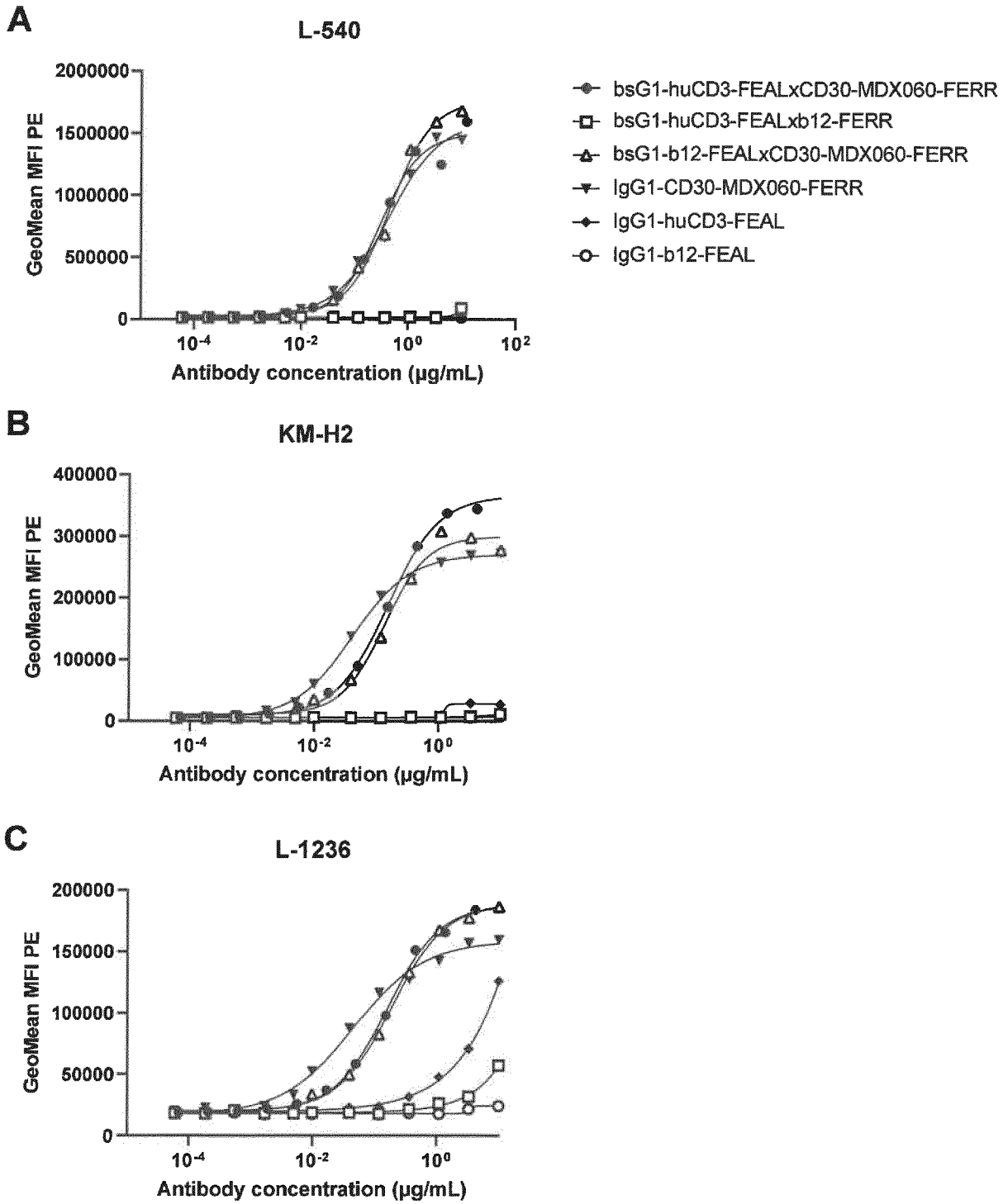

A
L-540
GeoMean MFI PE
2000000
1500000
1000000
500000
0
10-4
10-2
100
102
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL
B
KM-H2
GeoMean MFI PE
400000
300000
200000
100000
0
10-4
10-2
100
Antibody concentration (µg/mL)
C
L-1236
GeoMean MFI PE
200000
150000
100000
50000
0
10-4
10-2
100
Antibody concentration (µg/mL)

Figure 9

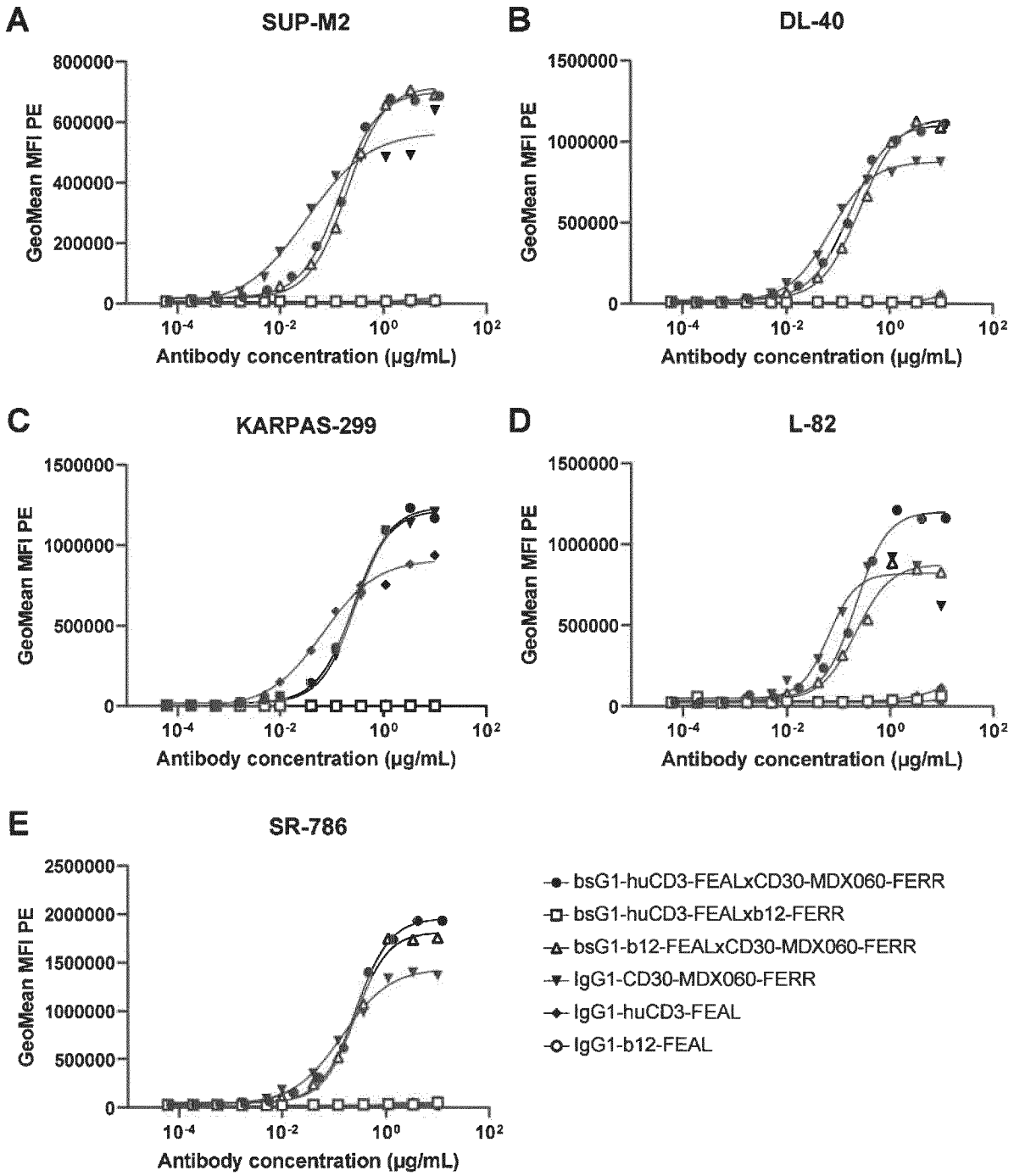

A
SUP-M2
B
DL-40
C
KARPAS-299
D
L-82
E
SR-786
GeoMean MFI PE
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 10

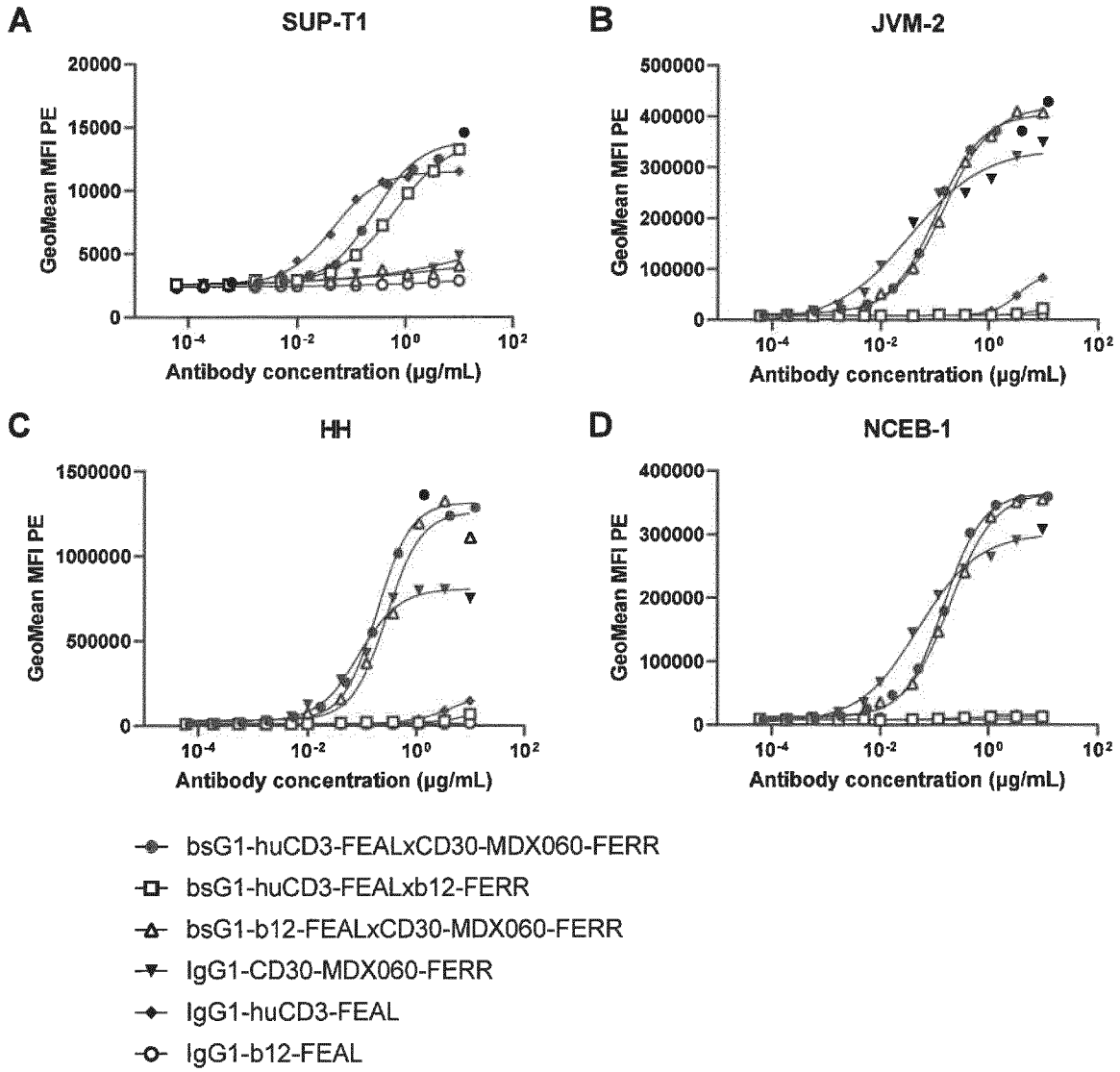

A
SUP-T1
GeoMean MFI PE
20000
15000
10000
5000
0
10-4
10-2
100
102
Antibody concentration (µg/mL)
B
JVM-2
GeoMean MFI PE
500000
400000
300000
200000
100000
0
10-4
10-2
100
102
Antibody concentration (µg/mL)
C
HH
GeoMean MFI PE
1500000
1000000
500000
0
10-4
10-2
100
102
Antibody concentration (µg/mL)
D
NCEB-1
GeoMean MFI PE
400000
300000
200000
100000
0
10-4
10-2
100
102
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 11

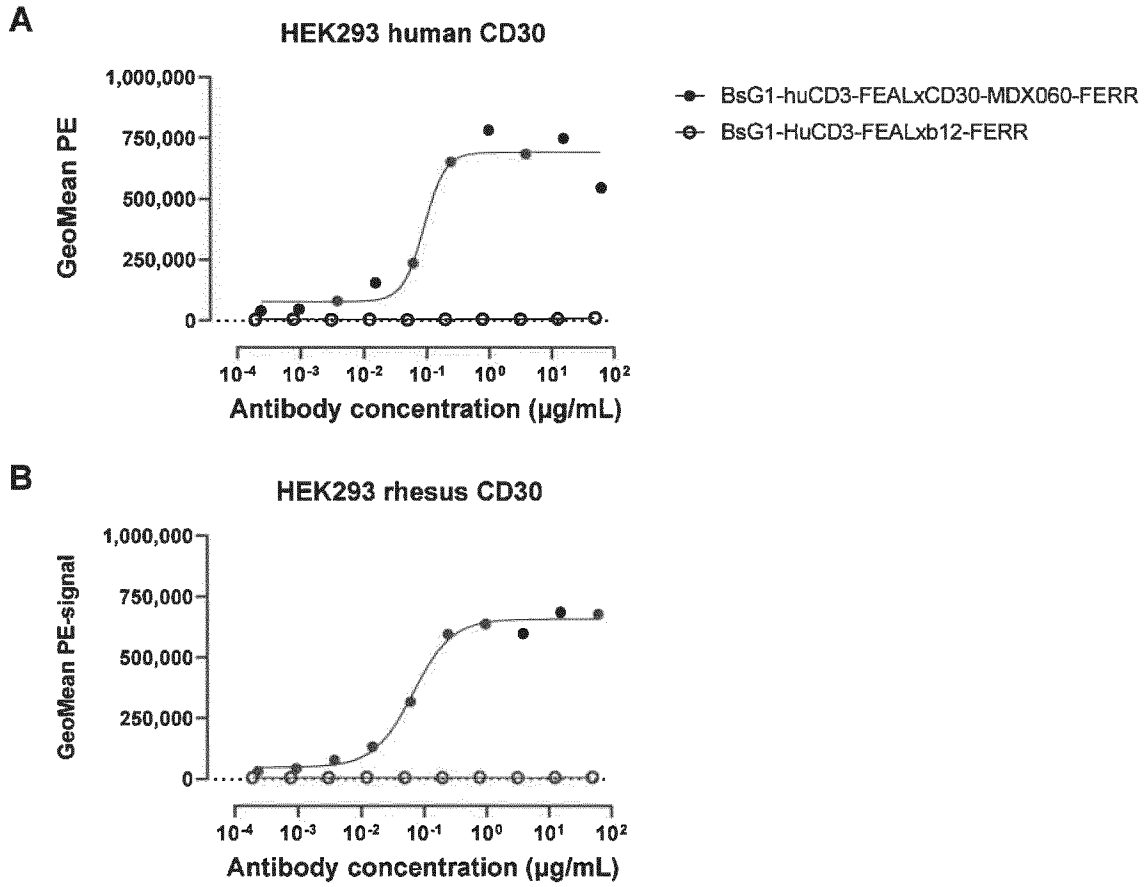
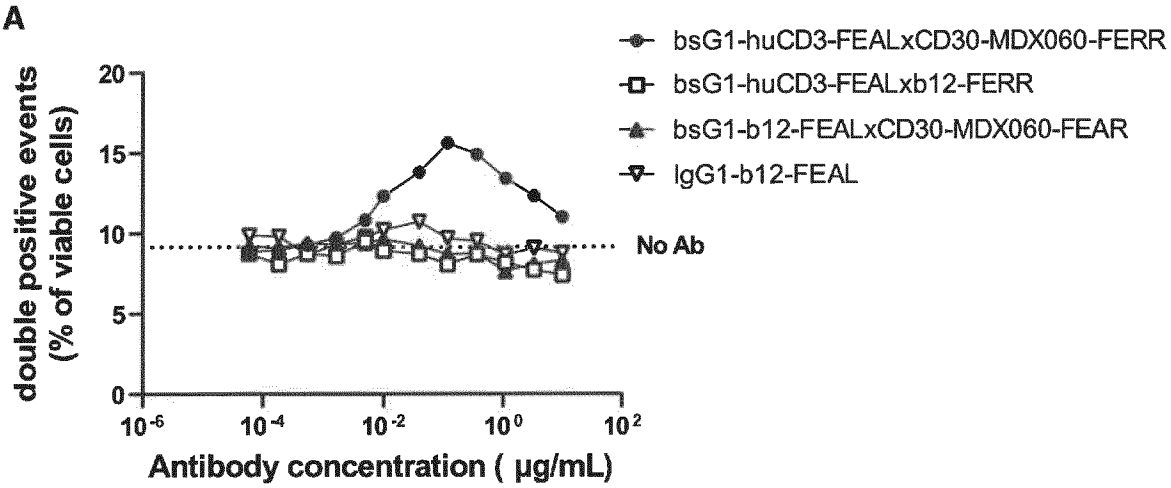

A
HEK293 human CD30
GeoMean PE
1,000,000
750,000
500,000
250,000
0
10-4 10-3 10-2 10-1 100 101 102
Antibody concentration (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FERR
BsG1-HuCD3-FEALxb12-FERR
B
HEK293 rhesus CD30
GeoMean PE-signal
1,000,000
750,000
500,000
250,000
0
10-4 10-3 10-2 10-1 100 101 102
Antibody concentration (µg/mL)

Figure 12

A


bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FEAR
IgG1-b12-FEAL
No Ab
double positive events
(% of viable cells)
20
15
10
5
0
10-6
10-4
10-2
100
102
Antibody concentration (µg/mL)

B

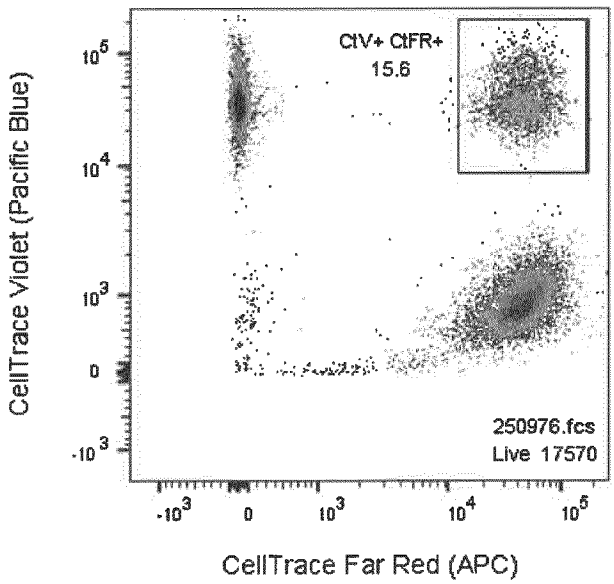

CtV+ CtFR+
15.6
250976.fcs
Live 17570
CellTrace Violet (Pacific Blue)
CellTrace Far Red (APC)
105
104
103
0
-103

Figure 13

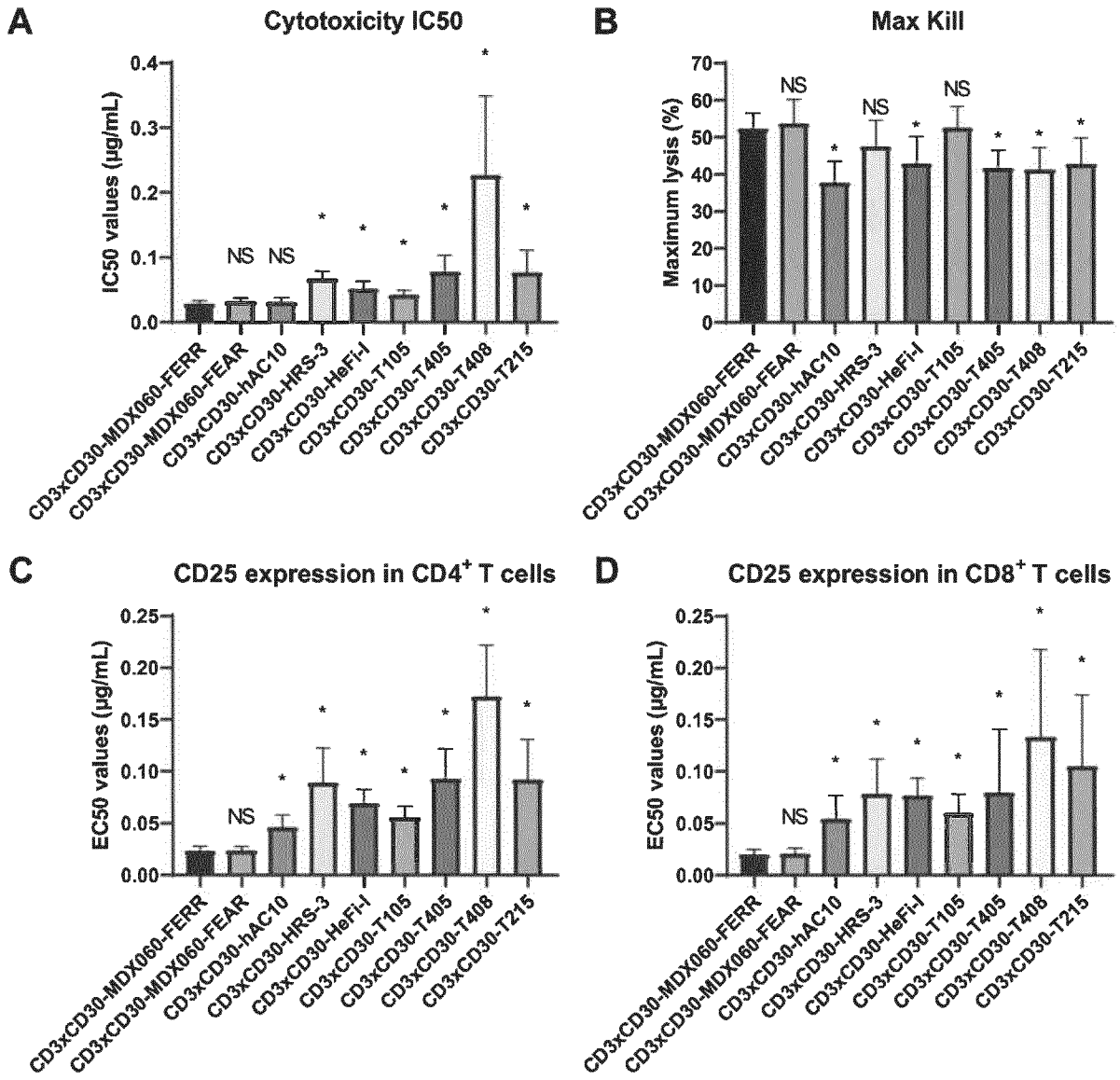

A
Cytotoxicity IC50
IC50 values (µg/mL)
0.4
0.3
0.2
0.1
0.0
NS
NS
*
CD3xCD30-MDX060-FERR
CD3xCD30-MDX060-FEAR
CD3xCD30-hAC10
CD3xCD30-HRS-3
CD3xCD30-HeFi-I
CD3xCD30-T105
CD3xCD30-T405
CD3xCD30-T408
CD3xCD30-T215
B
Max Kill
Maximum lysis (%)
70
60
50
40
30
20
10
0
NS
NS
NS
C
CD25 expression in CD4+ T cells
EC50 values (µg/mL)
0.25
0.20
0.15
0.10
0.05
0.00
D
CD25 expression in CD8+ T cells
EC50 values (µg/mL)

Figure 13 (continued)

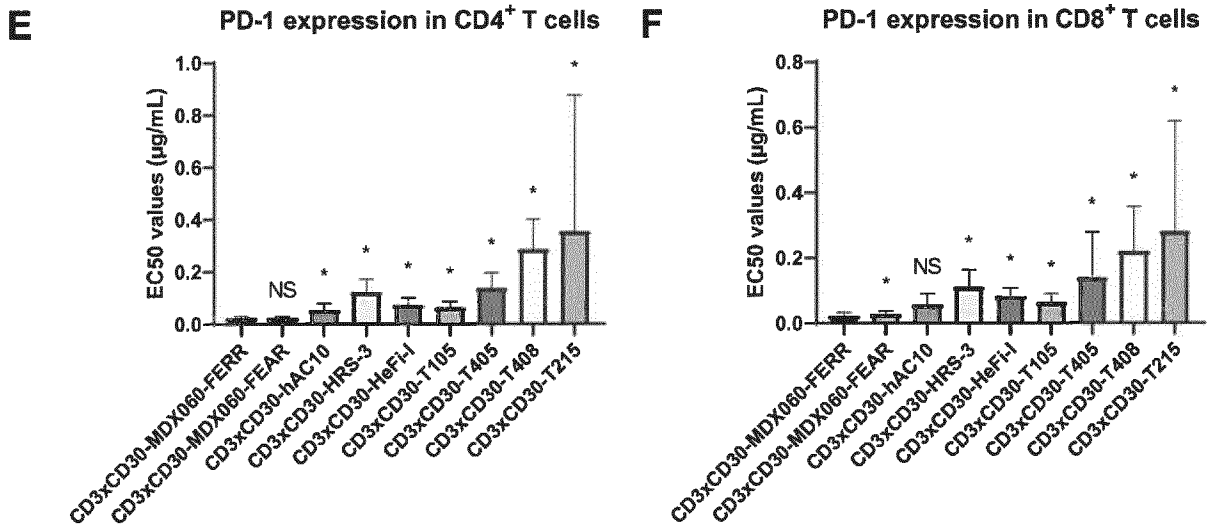


Figure 14

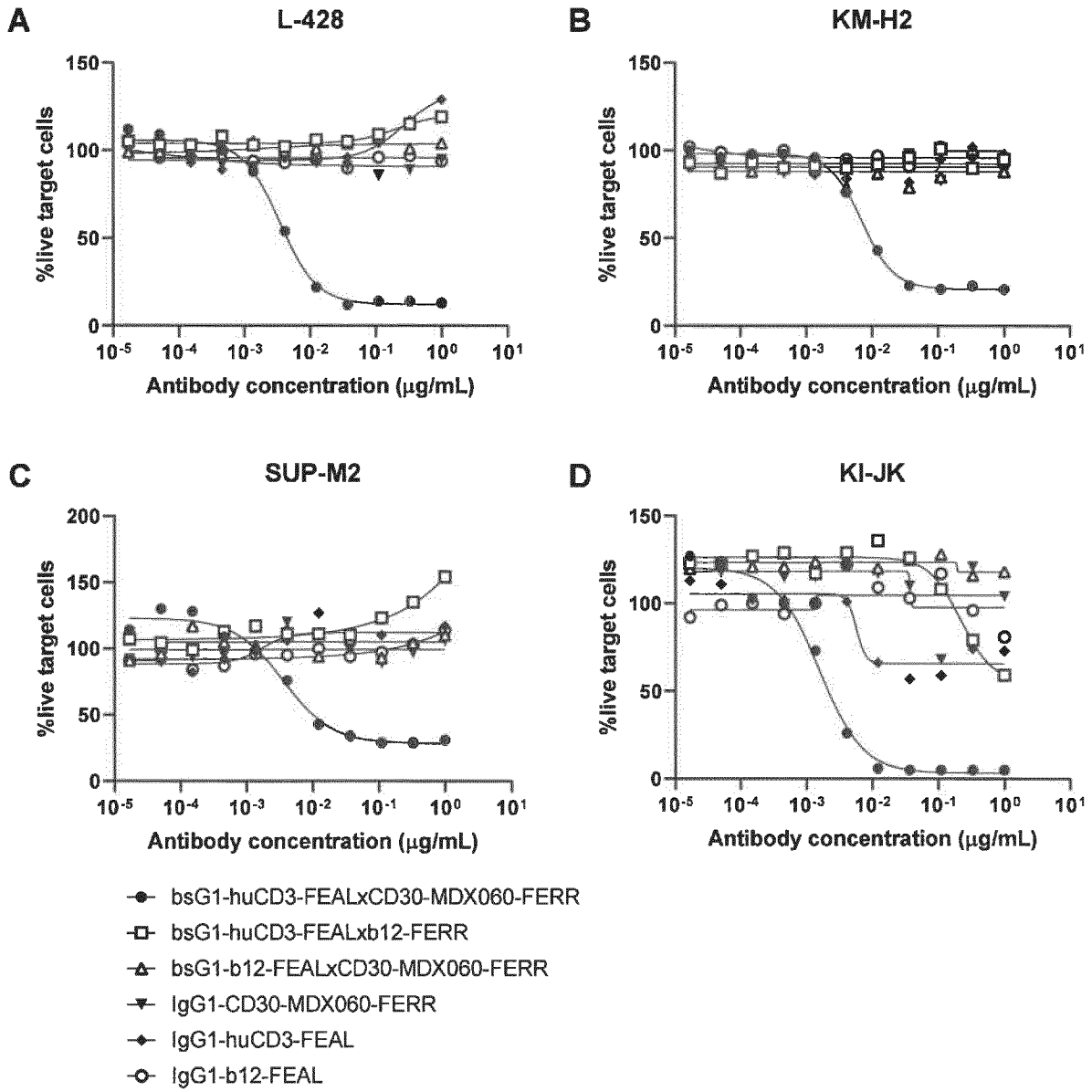

A
L-428
B
KM-H2
C
SUP-M2
D
KI-JK
%live target cells
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 15

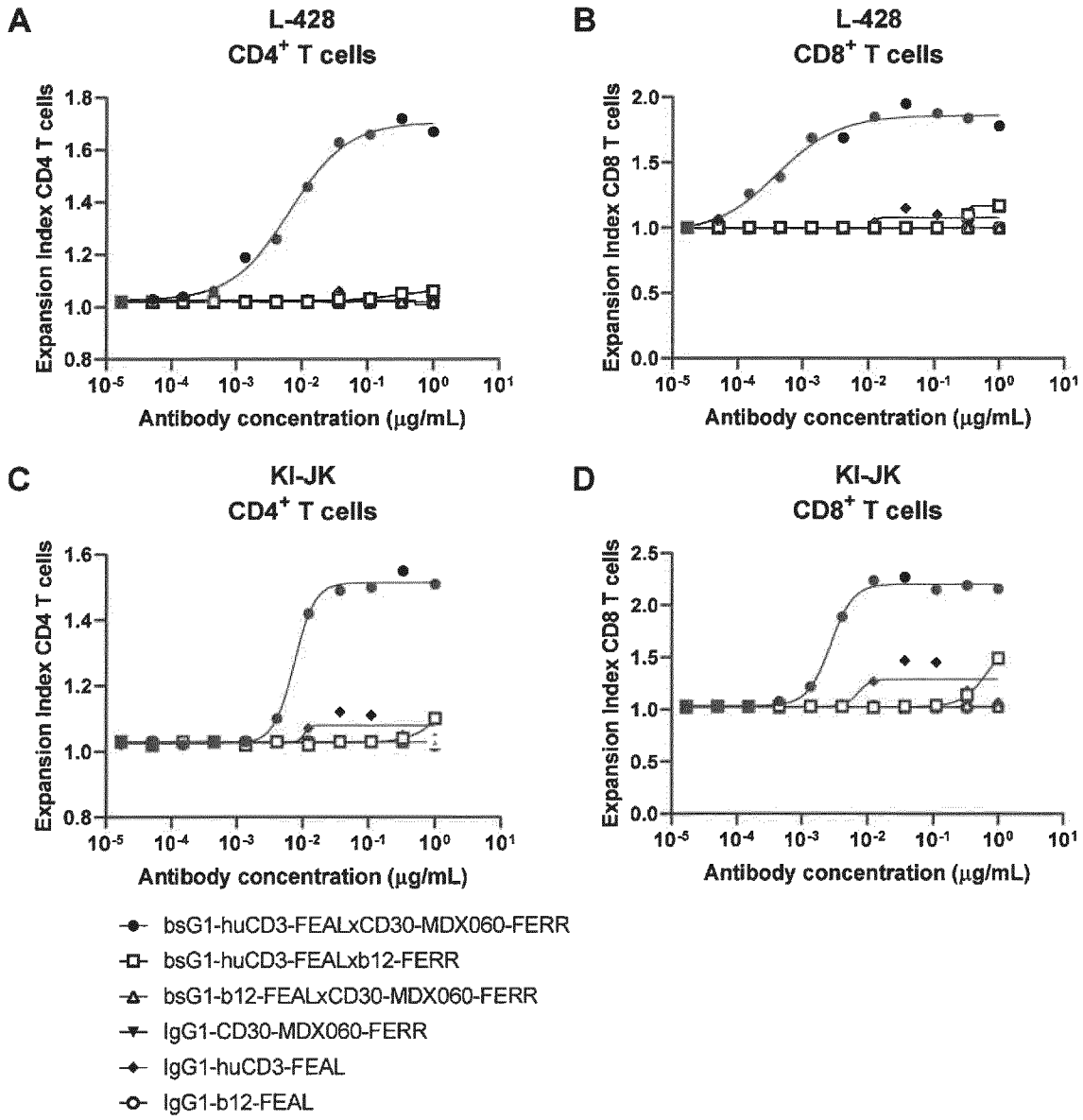

A
L-428
CD4+ T cells
Expansion Index CD4 T cells
Antibody concentration (µg/mL)
B
L-428
CD8+ T cells
Expansion Index CD8 T cells
Antibody concentration (µg/mL)
C
KI-JK
CD4+ T cells
Expansion Index CD4 T cells
Antibody concentration (µg/mL)
D
KI-JK
CD8+ T cells
Expansion Index CD8 T cells
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 16

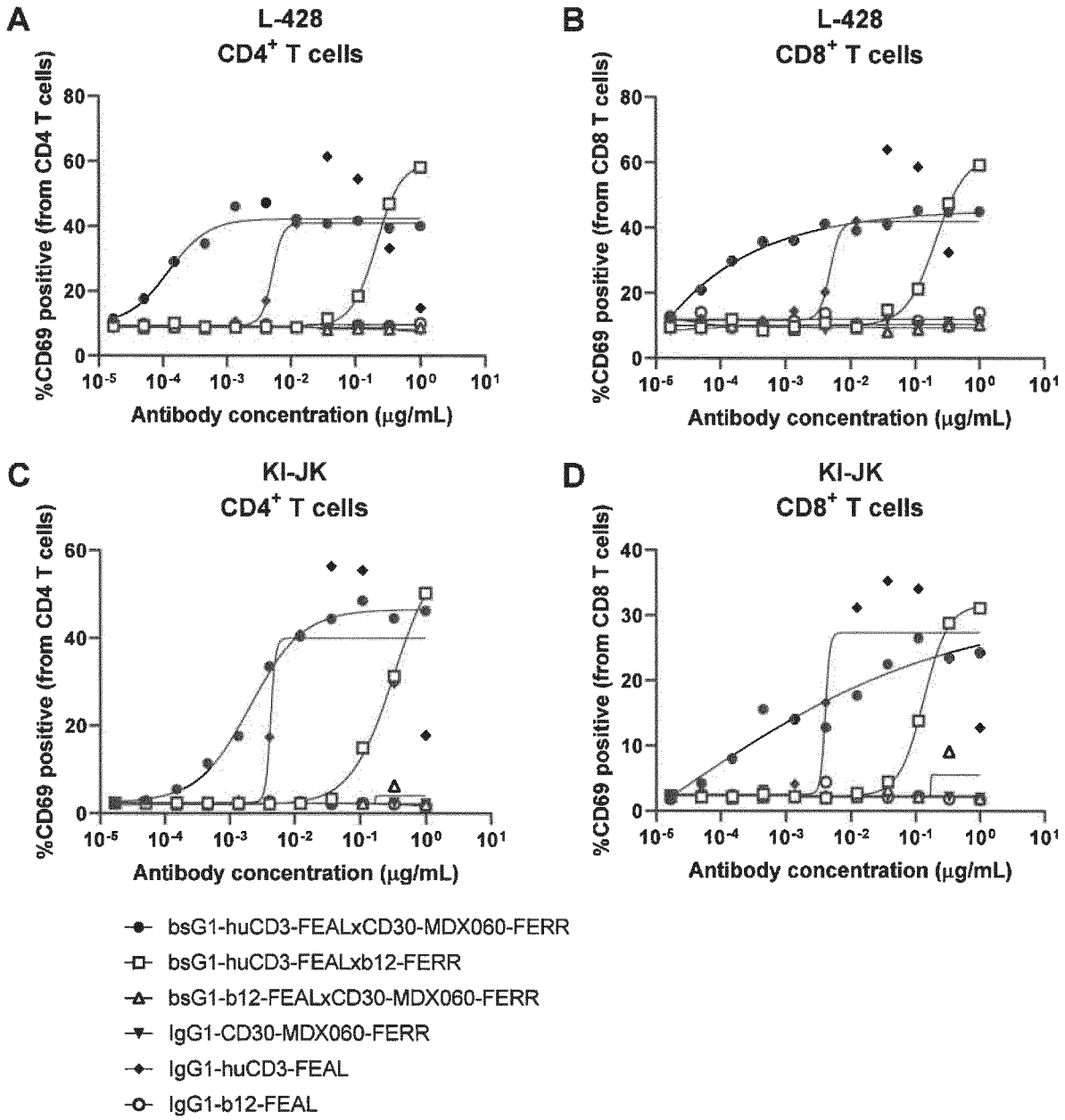

A
L-428
CD4+ T cells
%CD69 positive (from CD4 T cells)
Antibody concentration (µg/mL)
B
L-428
CD8+ T cells
%CD69 positive (from CD8 T cells)
Antibody concentration (µg/mL)
C
KI-JK
CD4+ T cells
%CD69 positive (from CD4 T cells)
Antibody concentration (µg/mL)
D
KI-JK
CD8+ T cells
%CD69 positive (from CD8 T cells)
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 17

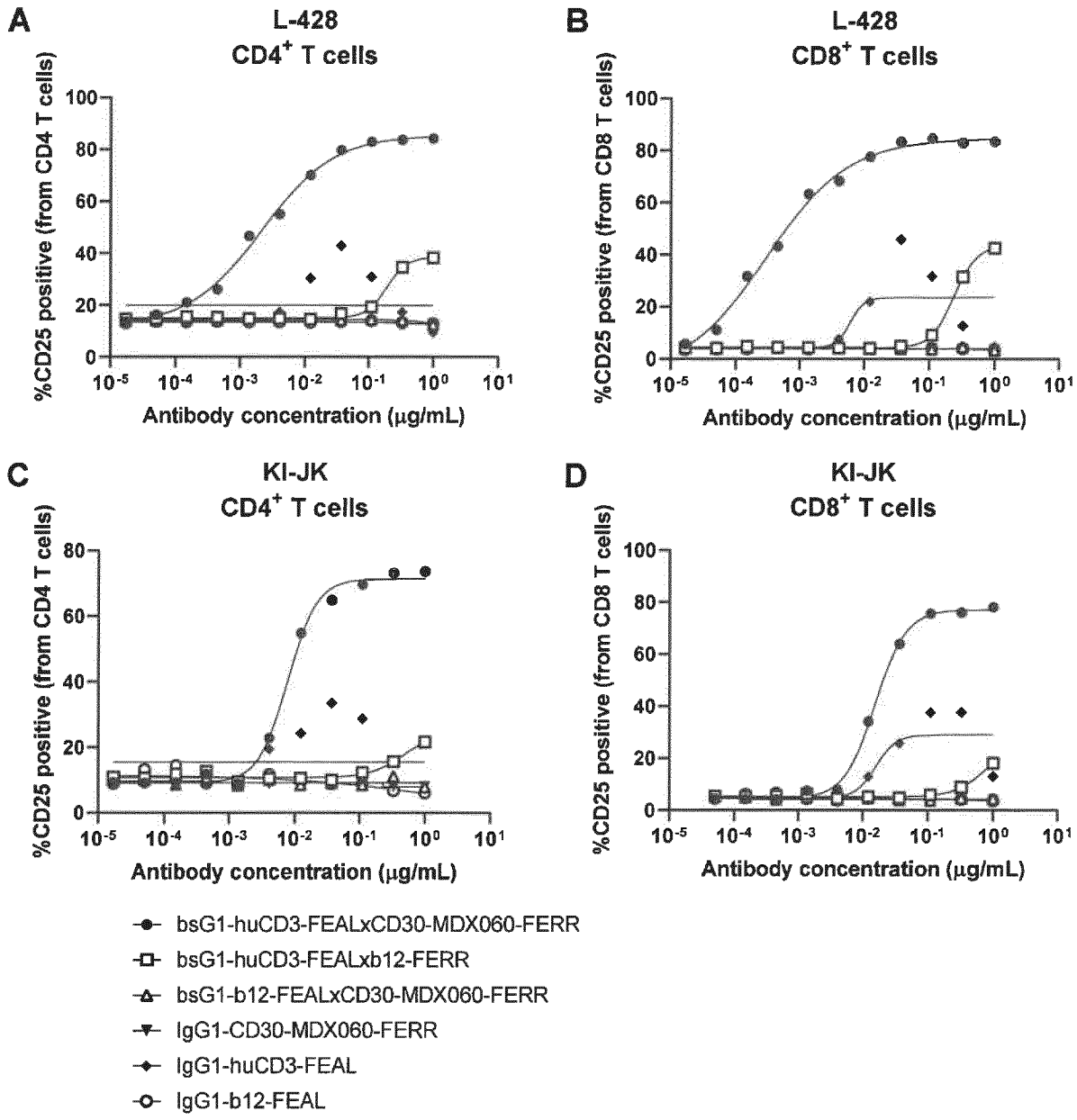

A
L-428
CD4+ T cells
%CD25 positive (from CD4 T cells)
Antibody concentration (µg/mL)
B
L-428
CD8+ T cells
%CD25 positive (from CD8 T cells)
Antibody concentration (µg/mL)
C
KI-JK
CD4+ T cells
%CD25 positive (from CD4 T cells)
Antibody concentration (µg/mL)
D
KI-JK
CD8+ T cells
%CD25 positive (from CD8 T cells)
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 18

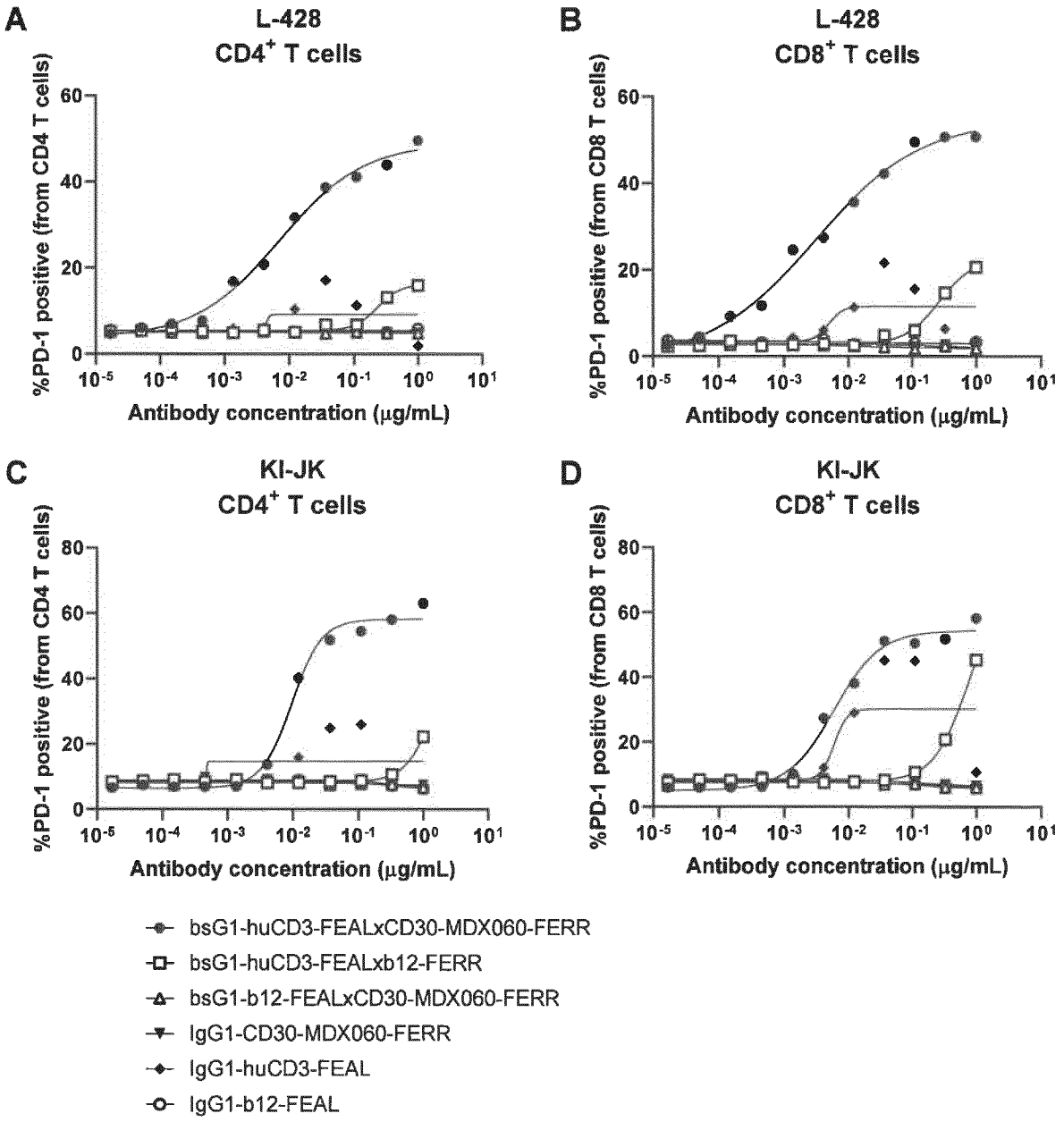

A
L-428
CD4+ T cells
%PD-1 positive (from CD4 T cells)
Antibody concentration (µg/mL)
B
L-428
CD8+ T cells
%PD-1 positive (from CD8 T cells)
Antibody concentration (µg/mL)
C
KI-JK
CD4+ T cells
%PD-1 positive (from CD4 T cells)
Antibody concentration (µg/mL)
D
KI-JK
CD8+ T cells
%PD-1 positive (from CD8 T cells)
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
bsG1-huCD3-FEALxb12-FERR
bsG1-b12-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-huCD3-FEAL
IgG1-b12-FEAL

Figure 19

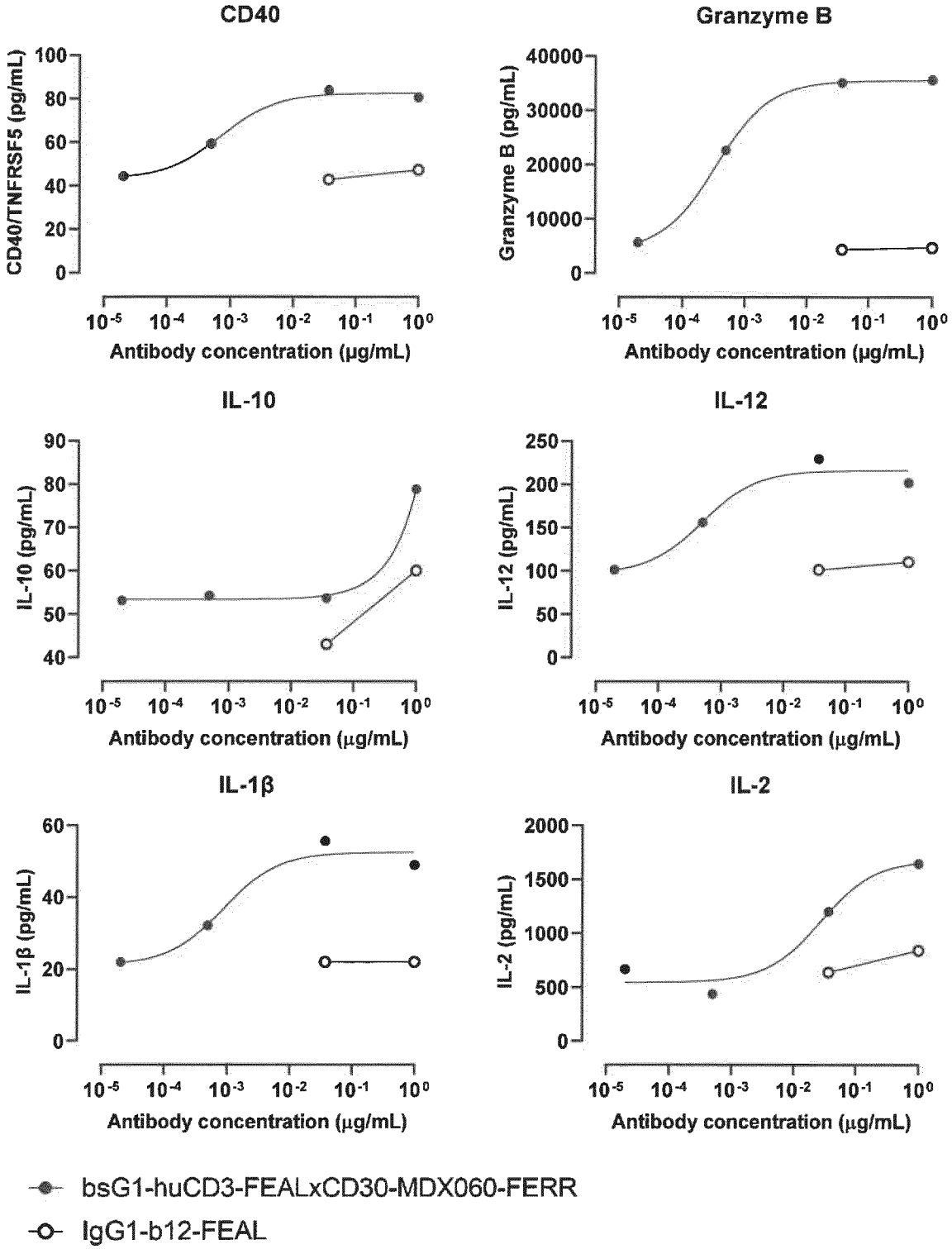

CD40
CD40/TNFRSF5 (pg/mL)
Antibody concentration (µg/mL)
Granzyme B
Granzyme B (pg/mL)
Antibody concentration (µg/mL)
IL-10
IL-10 (pg/mL)
Antibody concentration (µg/mL)
IL-12
IL-12 (pg/mL)
Antibody concentration (µg/mL)
IL-1β
IL-1β (pg/mL)
Antibody concentration (µg/mL)
IL-2
IL-2 (pg/mL)
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
IgG1-b12-FEAL

Figure 19 (continued)

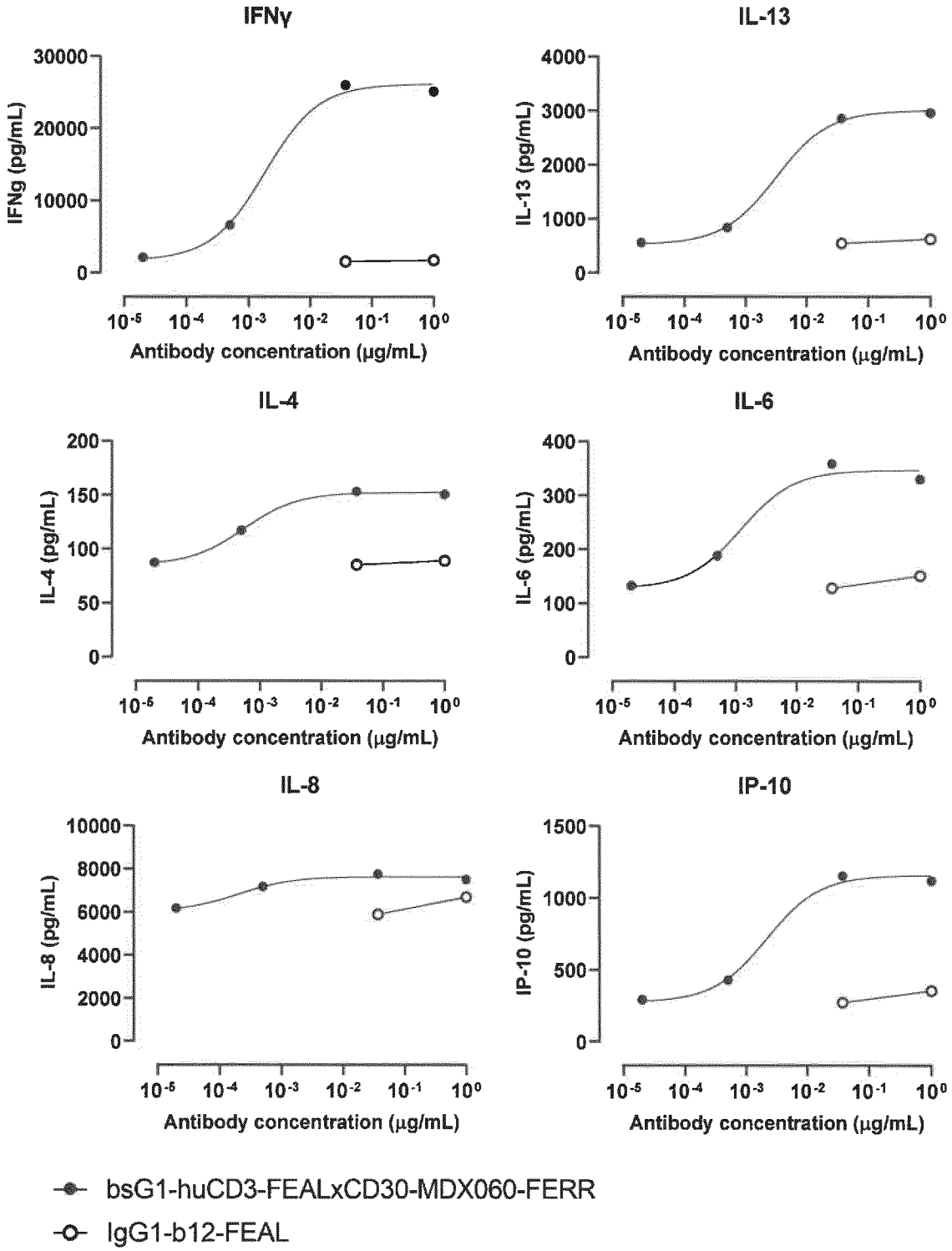


Figure 19 (continued)

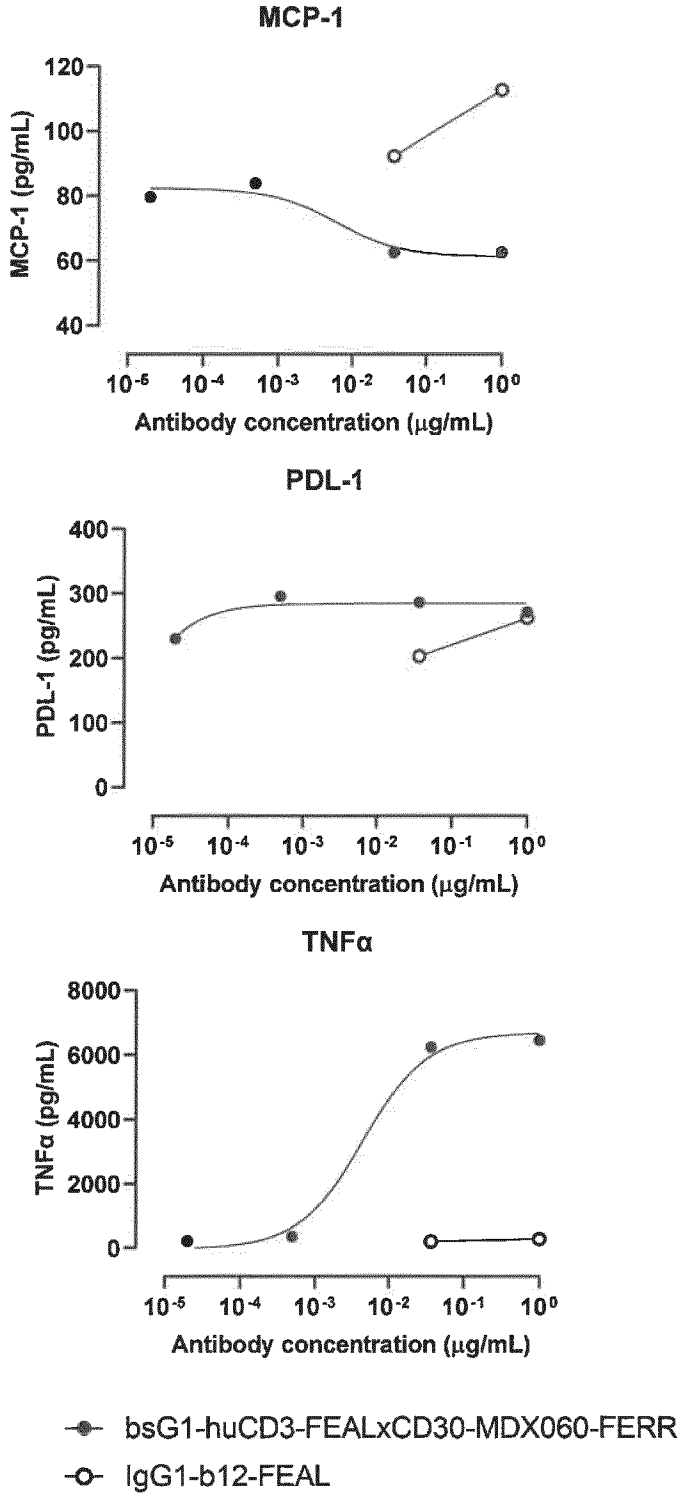


Figure 20

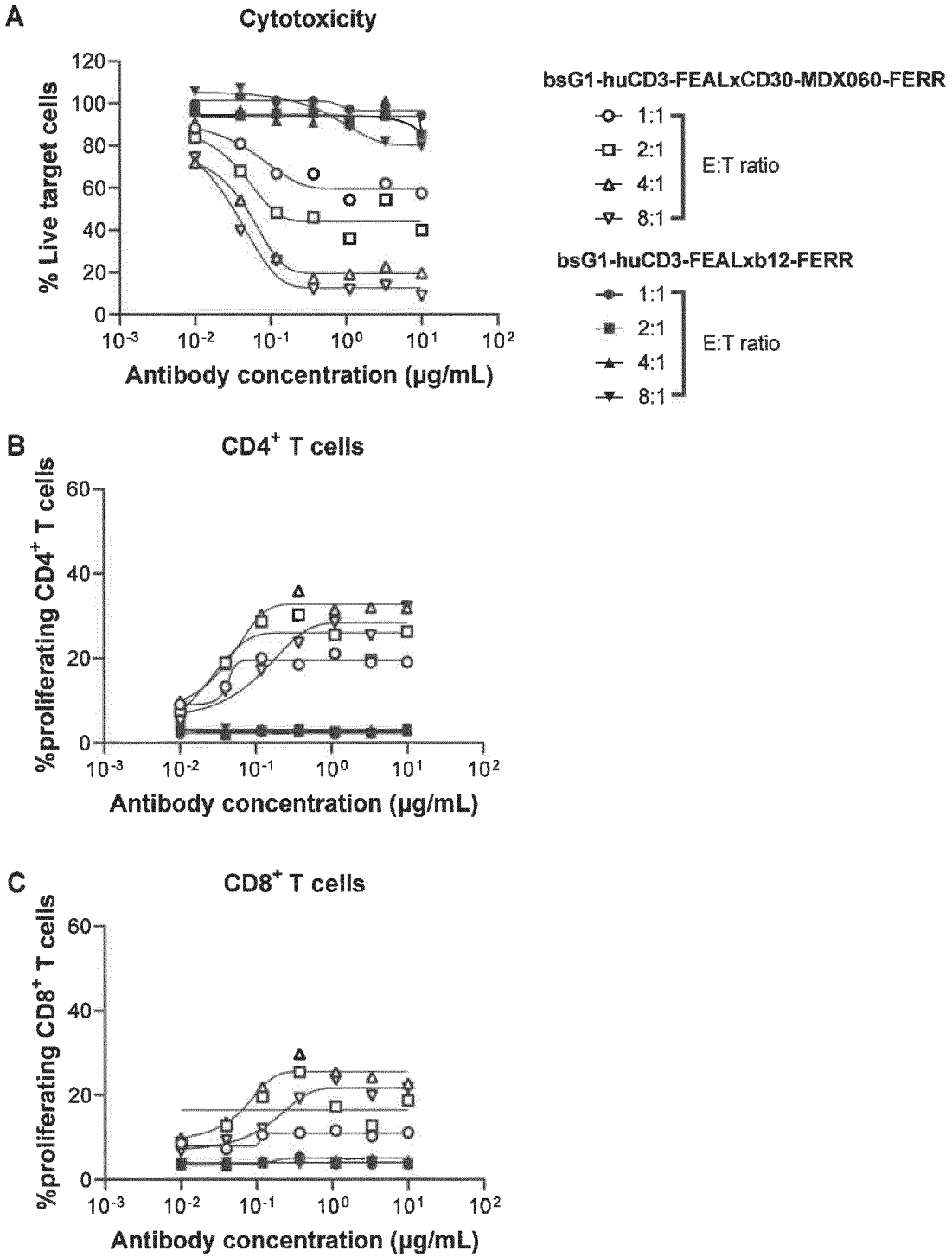

A
Cytotoxicity
% Live target cells
120
100
80
60
40
20
0
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
1:1
2:1
4:1
8:1
E:T ratio
bsG1-huCD3-FEALxb12-FERR
1:1
2:1
4:1
8:1
E:T ratio
B
CD4+ T cells
%proliferating CD4+ T cells
60
40
20
0
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/mL)
C
CD8+ T cells
%proliferating CD8+ T cells
60
40
20
0
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/mL)

Figure 21

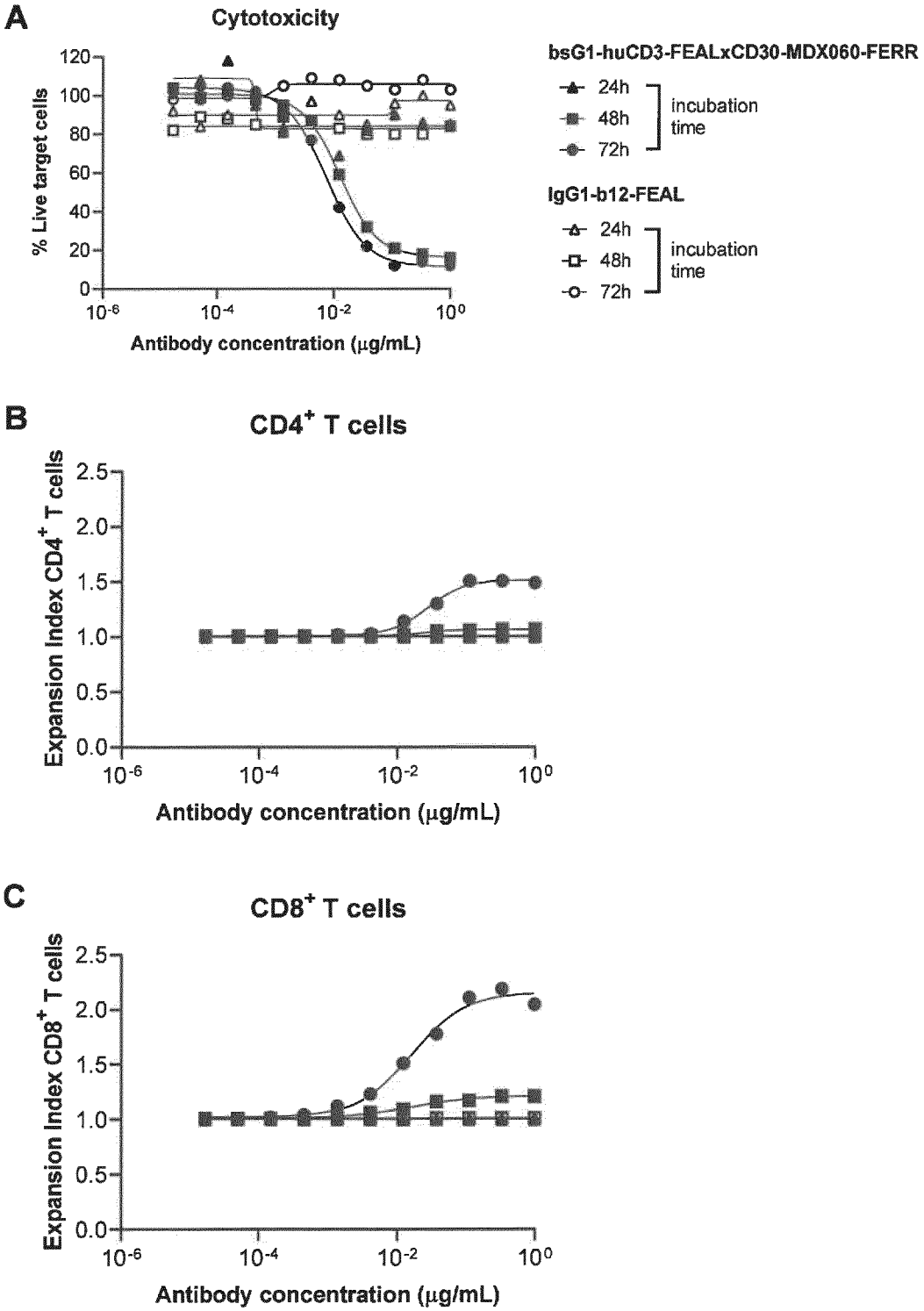

A
Cytotoxicity
% Live target cells
120
100
80
60
40
20
0
10-6
10-4
10-2
100
Antibody concentration (µg/mL)
bsG1-huCD3-FEALxCD30-MDX060-FERR
24h
48h
72h
incubation time
IgG1-b12-FEAL
24h
48h
72h
incubation time
B
CD4+ T cells
Expansion Index CD4+ T cells
2.5
2.0
1.5
1.0
0.5
0.0
10-6
10-4
10-2
100
Antibody concentration (µg/mL)
C
CD8+ T cells
Expansion Index CD8+ T cells
2.5
2.0
1.5
1.0
0.5
0.0
10-6
10-4
10-2
100
Antibody concentration (µg/mL)

Figure 22

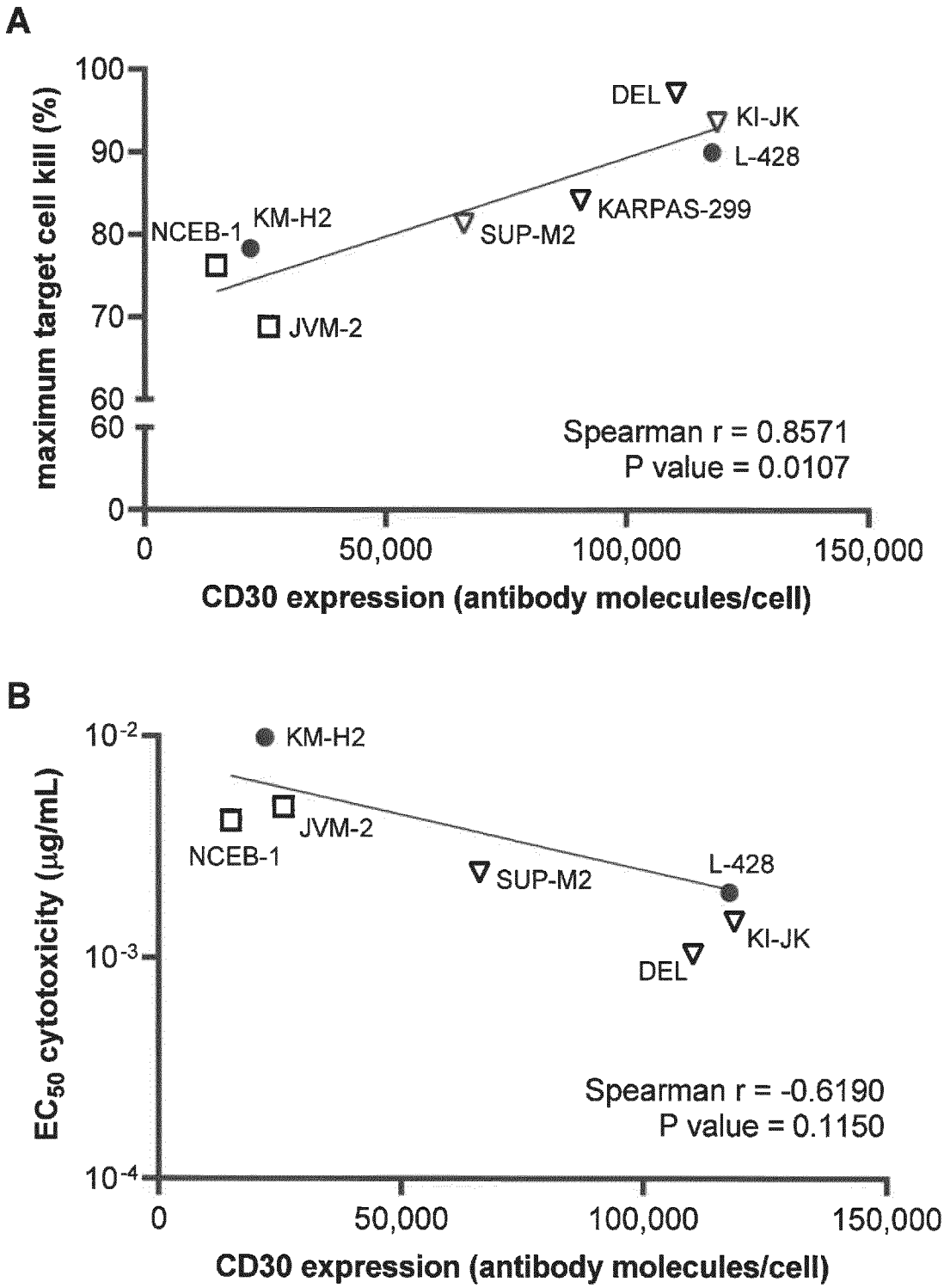

A
maximum target cell kill (%)
100
90
80
70
60
60
0
DEL
KI-JK
L-428
KARPAS-299
SUP-M2
NCEB-1
KM-H2
JVM-2
Spearman r = 0.8571
P value = 0.0107
0
50,000
100,000
150,000
CD30 expression (antibody molecules/cell)
B
EC50 cytotoxicity (µg/mL)
10-2
10-3
10-4
KM-H2
JVM-2
NCEB-1
SUP-M2
L-428
KI-JK
DEL
Spearman r = -0.6190
P value = 0.1150
0
50,000
100,000
150,000
CD30 expression (antibody molecules/cell)

Figure 23

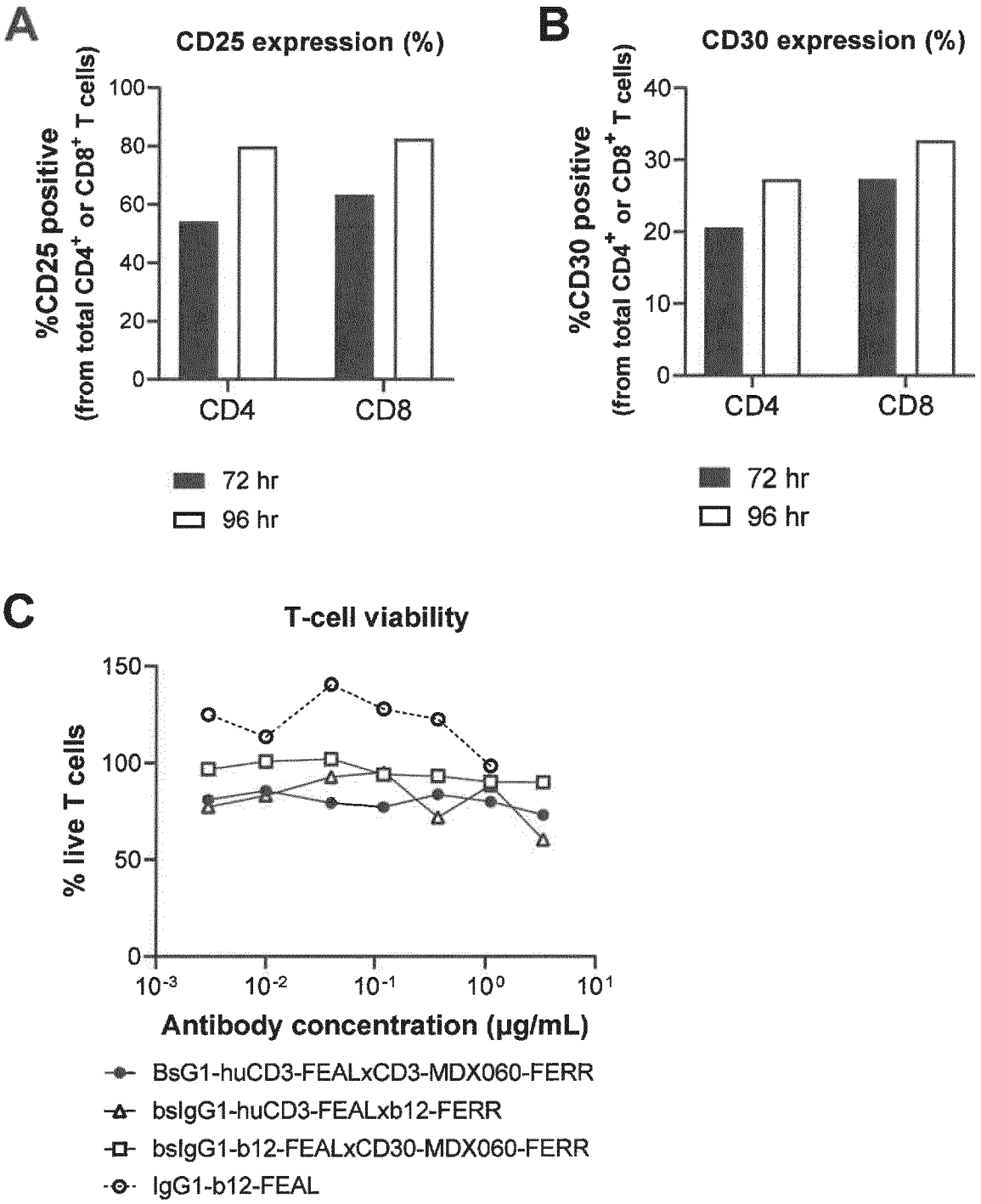

A
CD25 expression (%)
%CD25 positive
(from total CD4+ or CD8+ T cells)
100
80
60
40
20
0
CD4
CD8
72 hr
96 hr
B
CD30 expression (%)
%CD30 positive
(from total CD4+ or CD8+ T cells)
40
30
20
10
0
CD4
CD8
72 hr
96 hr
C
T-cell viability
% live T cells
150
100
50
0
10-3
10-2
10-1
100
101
Antibody concentration (µg/mL)
BsG1-huCD3-FEALxCD3-MDX060-FERR
bsIgG1-huCD3-FEALxb12-FERR
bsIgG1-b12-FEALxCD30-MDX060-FERR
IgG1-b12-FEAL

Figure 24

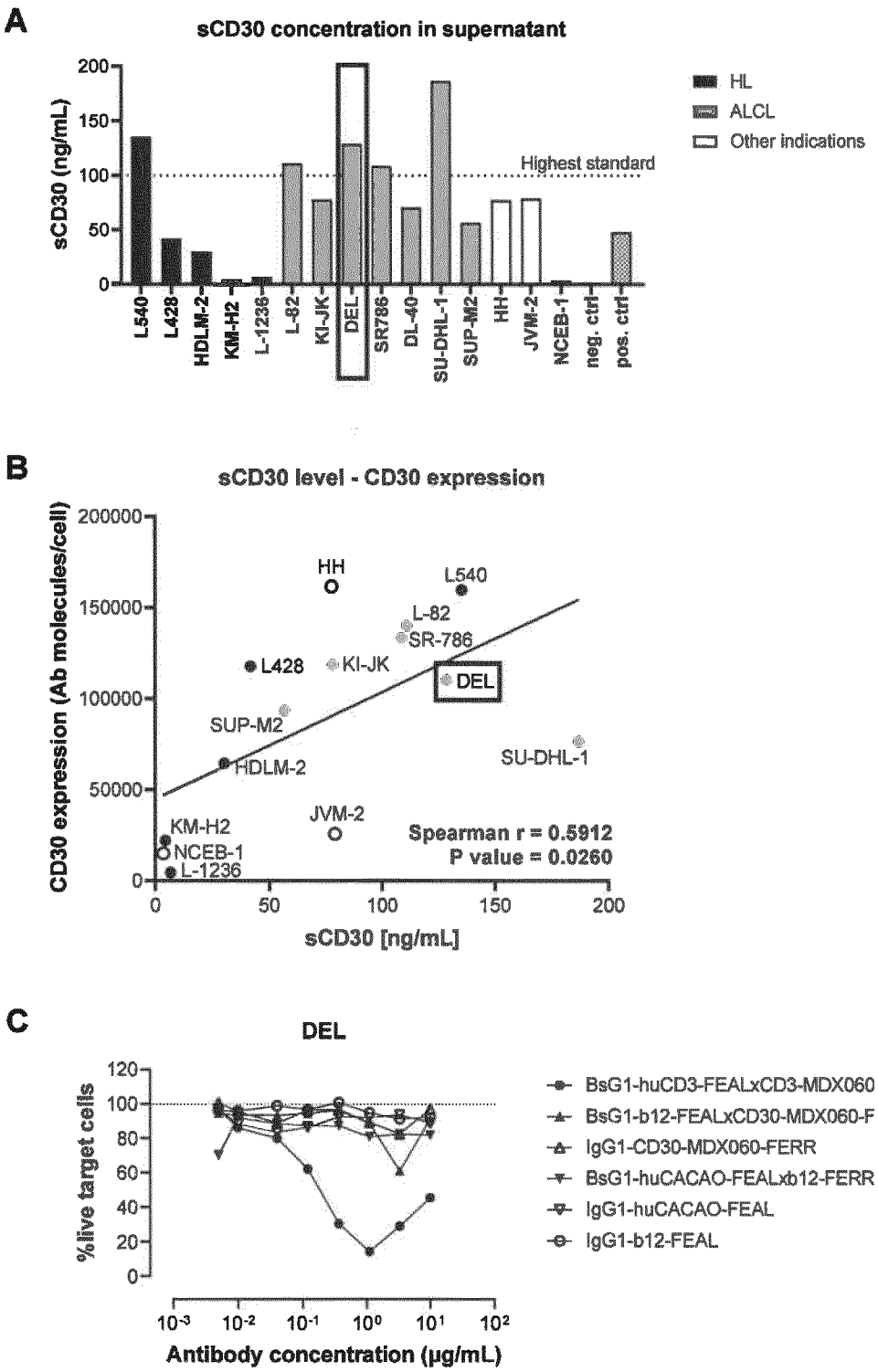

A
sCD30 concentration in supernatant
sCD30 (ng/mL)
200
150
100
50
0
Highest standard
HL
ALCL
Other indications
L540
L428
HDLM-2
KM-H2
L-1236
L-82
KI-JK
DEL
SR786
DL-40
SU-DHL-1
SUP-M2
HH
JVM-2
NCEB-1
neg. ctrl
pos. ctrl
B
sCD30 level - CD30 expression
CD30 expression (Ab molecules/cell)
200000
150000
100000
50000
0
HH
L540
L-82
SR-786
L428
KI-JK
DEL
SUP-M2
HDLM-2
SU-DHL-1
JVM-2
KM-H2
NCEB-1
L-1236
Spearman r = 0.5912
P value = 0.0260
0
50
100
150
200
sCD30 [ng/mL]
C
DEL
%live target cells
120
100
80
60
40
20
0
BsG1-huCD3-FEALxCD3-MDX060
BsG1-b12-FEALxCD30-MDX060-F
IgG1-CD30-MDX060-FERR
BsG1-huCACAO-FEALxb12-FERR
IgG1-huCACAO-FEAL
IgG1-b12-FEAL
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/mL)

Figure 25

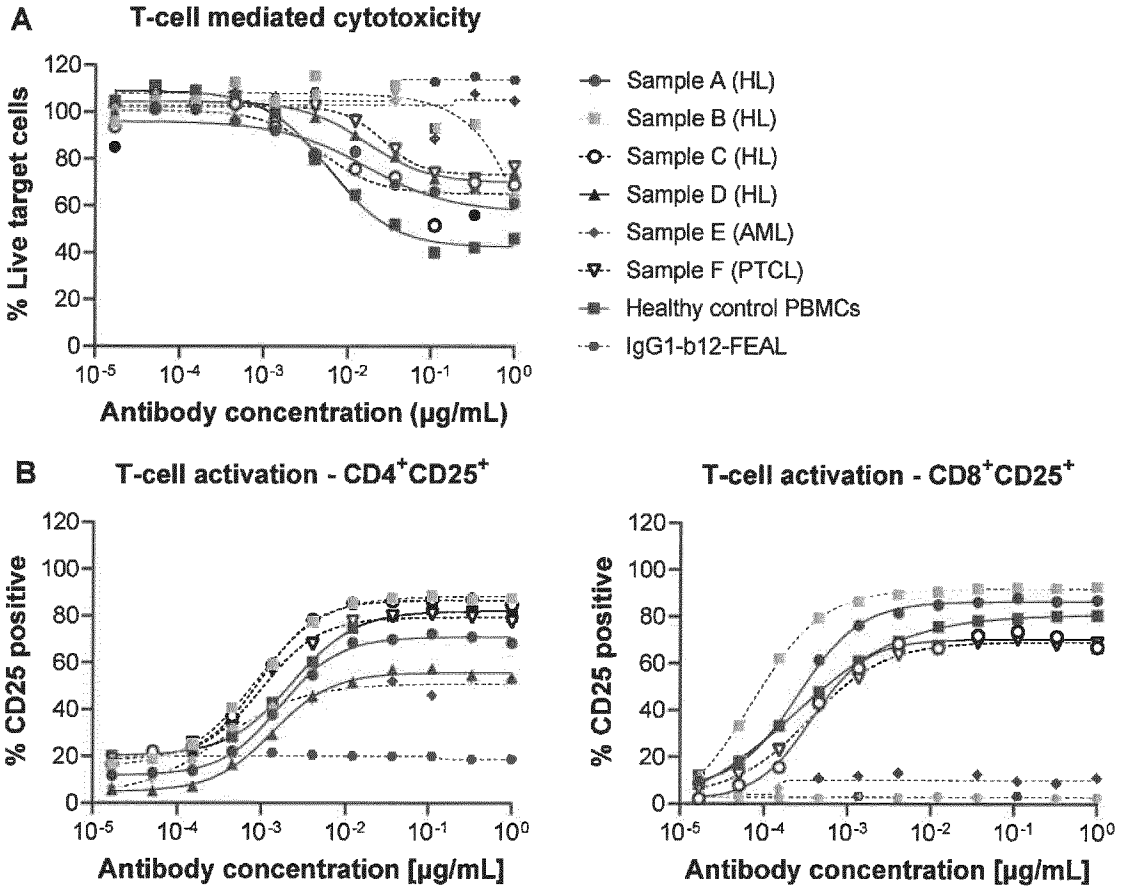

A
T-cell mediated cytotoxicity
% Live target cells
Antibody concentration (µg/mL)
Sample A (HL)
Sample B (HL)
Sample C (HL)
Sample D (HL)
Sample E (AML)
Sample F (PTCL)
Healthy control PBMCs
IgG1-b12-FEAL
B
T-cell activation - CD4+CD25+
T-cell activation - CD8+CD25+
% CD25 positive
Antibody concentration [µg/mL]

Figure 25 (continued)

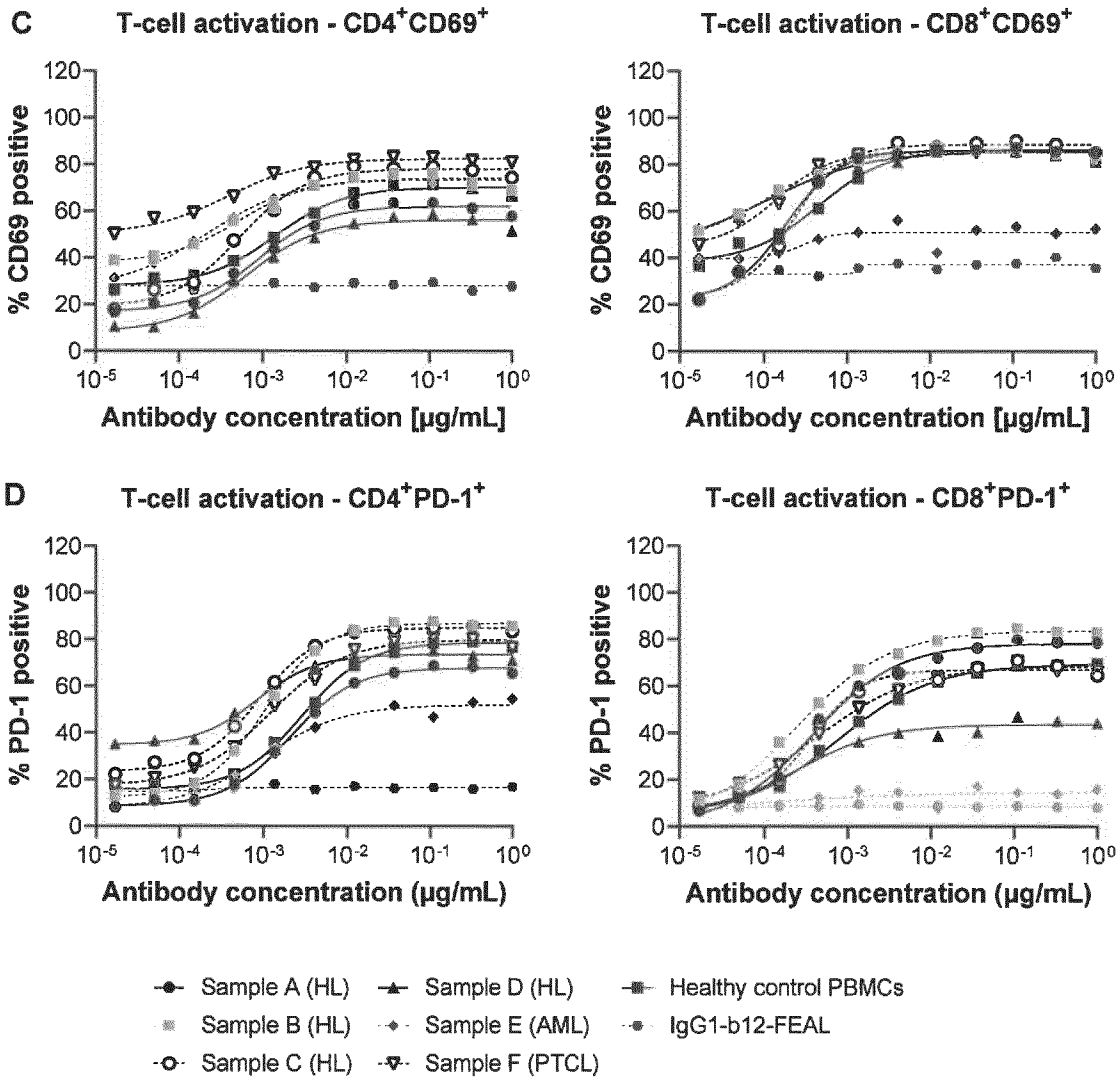
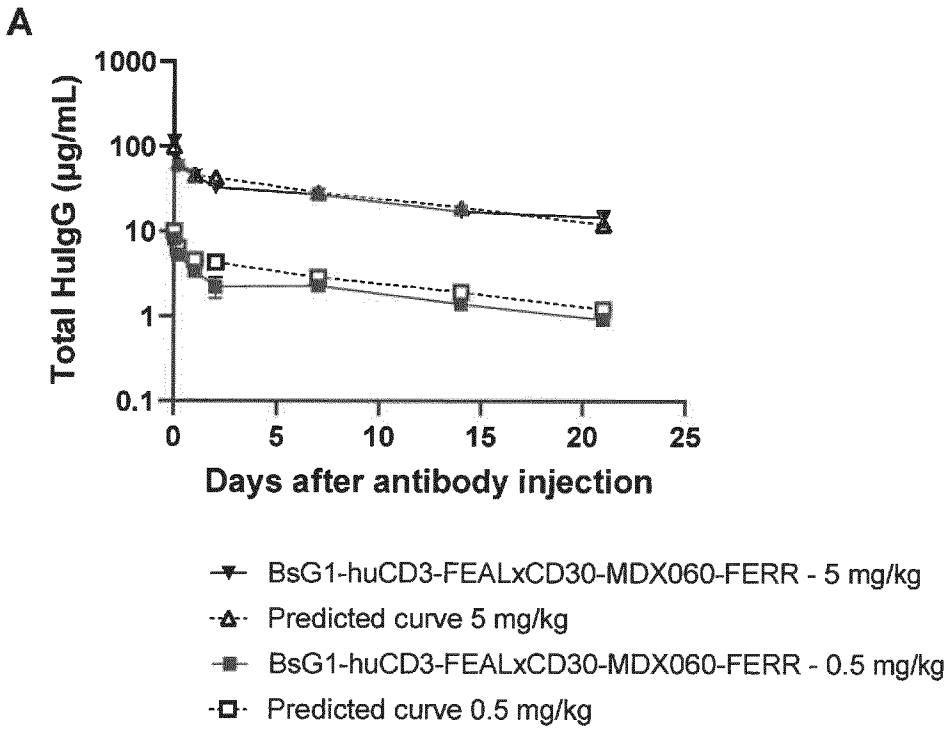


Figure 26


A
Total HuIgG (µg/mL)
1000
100
10
1
0.1
0
5
10
15
20
25
Days after antibody injection
BsG1-huCD3-FEALxCD30-MDX060-FERR - 5 mg/kg
Predicted curve 5 mg/kg
BsG1-huCD3-FEALxCD30-MDX060-FERR - 0.5 mg/kg
Predicted curve 0.5 mg/kg

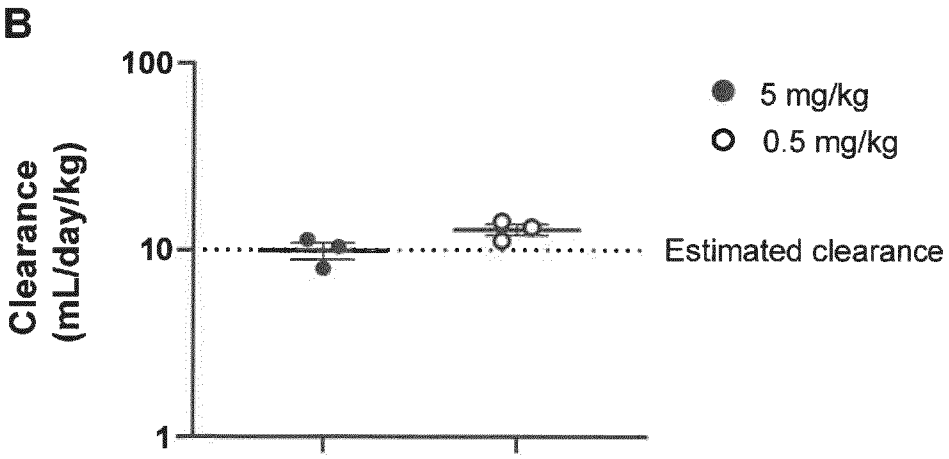
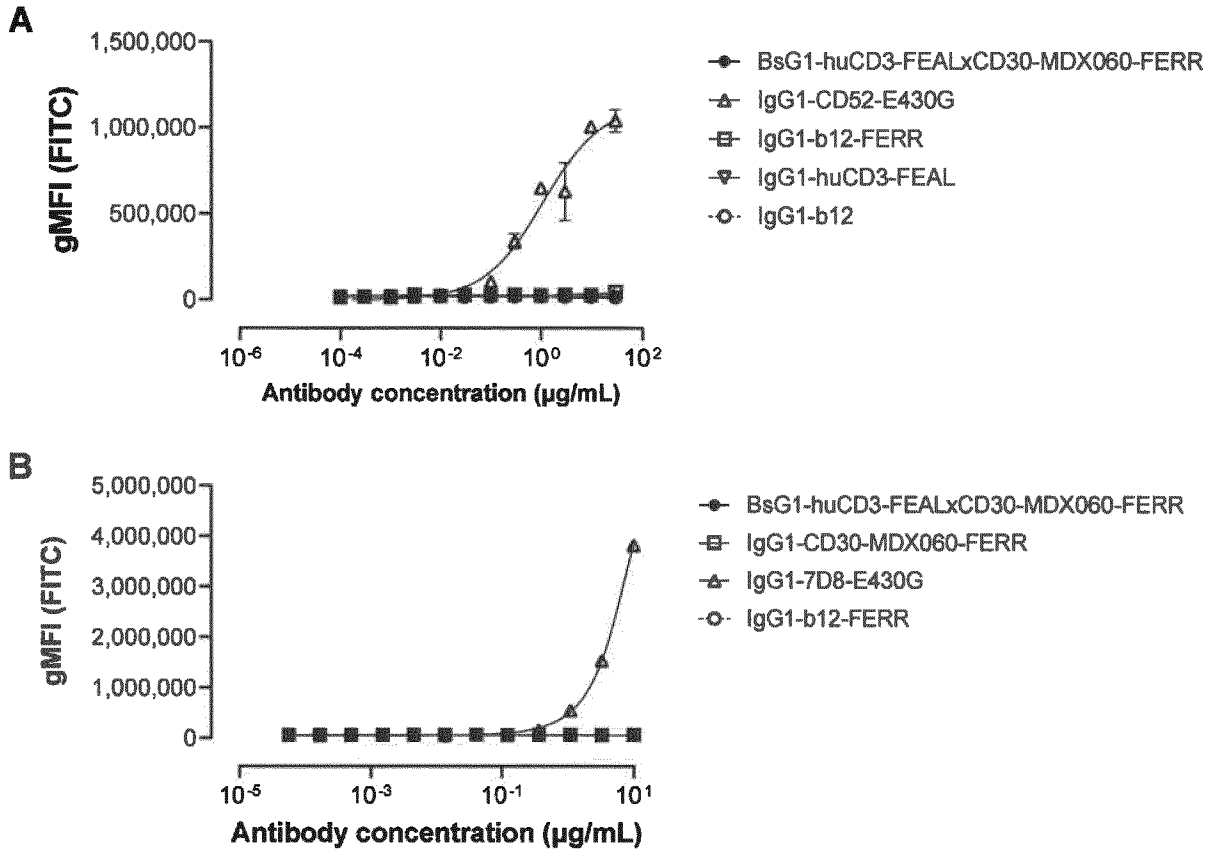

B
Clearance (mL/day/kg)
100
10
1
5 mg/kg
0.5 mg/kg
Estimated clearance

Figure 27


A
1,500,000
1,000,000
500,000
0
gMFI (FITC)
10-6
10-4
10-2
100
102
Antibody concentration (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FERR
IgG1-CD52-E430G
IgG1-b12-FERR
IgG1-huCD3-FEAL
IgG1-b12
B
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
gMFI (FITC)
10-5
10-3
10-1
101
Antibody concentration (µg/mL)
BsG1-huCD3-FEALxCD30-MDX060-FERR
IgG1-CD30-MDX060-FERR
IgG1-7D8-E430G
IgG1-b12-FERR

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/077817

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/28 A61K39/395 A61P35/00 A61K39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/119567 A2 (MICROMET AG [DE]; EBERT EVELYN [DE] ET AL.) 9 October 2008 (2008-10-09) cited in the application See e.g. SEQ ID NOs: 1113 and 1118; Example 29 from pages 176-183; Figures 46 and 47	1-48, 58-60

Y	US 2019/284278 A1 (RADEMAKER RIK [NL] ET AL) 19 September 2019 (2019-09-19) See e.g. SEQ ID NOs: 1,2, 3, 6, 7, 176; paragraphs 846-855, 859, 861, 715 and 716; Table 1 VL CDR2; claim 29(e)	1-48, 58-60

	-/--	

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 2022	30/01/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Valcárcel, Rafael

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2020/239579 A1 (ALTINTAS ISIL [DK] ET AL) 30 July 2020 (2020-07-30) See e.g. SEQ ID NOs: 26, 27, 28, 30, 31 and 45; Examples 3 and 12; paragraphs 239-256 and 291; claim 72; sequence GTN of the VL CDR2 of huCDR-L1 antibody in the sequence listing -----	1-48, 58-60
X	WO 2020/068774 A1 (MEDICAL COLLEGE WISCONSIN INC [US] ET AL.) 2 April 2020 (2020-04-02)	1-48, 58-60
Y	See e.g. Example 2 -----	1-48, 58-60
X	OLDHAM ROBYN A A ET AL: "T Cells Armed with Novel Anti-CD30/Anti-CD3 Bispecific Antibodies for Immunotherapy of CD30+Malignancies", MOLECULAR THERAPY, vol. 27, no. 4, Suppl. 1, 22 April 2019 (2019-04-22), page 387, XP009536442, & 22ND ANNUAL MEETING OF THE AMERICAN-SOCIETY-OF-GENE-AND-CELL-THERAPY (ASGCT); WASHINGTON, DC, USA; APRIL 29 -MAY 02, 2019	1-48, 58-60
Y	abstract -----	1-48, 58-60
X	SCHWARTZ C L ET AL: "Development of an A-CD30 bispecific antibody immunotherapy for Hodgkin lymphoma", HEMASPHERE 20181001 EMBASE CONFERENCE ABSTRACTS NLD, vol. 2, no. Supplement 1, 1 October 2018 (2018-10-01), XP009536443, ISSN: 2572-9241	1-48, 58-60
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Y	US 2017/073421 A1 (KJAERGAARD KRISTIAN [DK] ET AL) 16 March 2017 (2017-03-16) See e.g. paragraph 187, section (e) -----	1-48, 58-60
Y	US 2021/163594 A1 (BAILEY LUCAS [CN] ET AL) 3 June 2021 (2021-06-03) See e.g. examples 1-5 on pages 39-41 -----	1-48, 58-60
2 Y	WO 2006/105062 A2 (DIVERSA CORP [US]; HANSEN GENEVIEVE [US]; NESLUND GERALD [US]) 5 October 2006 (2006-10-05) See e.g. Figure 1 -----	1-48, 58-60

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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/006217 A1 (GENMAB BV [NL]) 9 January 2014 (2014-01-09) See e.g. page 43, lines 7 and 8; page 43, 7th paragraph	1-48, 58-60
Y	US 2021/009718 A1 (AMBROGELLY ALEXANDRE [US] ET AL) 14 January 2021 (2021-01-14) See e.g. paragraph 106	1-48, 58-60
Y	WO 2019/025545 A1 (GENMAB AS [DK]; BIONTECH AG [DE]) 7 February 2019 (2019-02-07) See e.g. Example 13	1-48, 58-60
Y	CHRISTIAN KLEIN ET AL: "Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies", MABS, vol. 4, no. 6, 1 November 2012 (2012-11-01), pages 653-663, XP055106060, ISSN: 1942-0862, DOI: 10.4161/mabs.21379 See e.g. Figures 2 and 5	1-48, 58-60
Y	DENNIS R. GOULET ET AL: "Kinetic mechanism of controlled Fab-arm exchange for the formation of bispecific immunoglobulin G1 antibodies", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 293, no. 2, 17 November 2017 (2017-11-17), pages 651-661, XP055549625, US ISSN: 0021-9258, DOI: 10.1074/jbc.RA117.000303 See e.g. the abstract	1-48, 58-60

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

1-48, 58-60

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-48, 58-60

   A multispecific antibody as defined in claim 1. A nucleic acid construct or a combination of nucleic acid constructs encoding said multispecific antibody. Expression vector, delivery vehicle, recombinant host cell, composition as defined in claims 28-36. The multispecific antibody for the uses as defined in claims 37-43. The above products that have been administered as defined in claim 44. Methods for producing the multispecific antibody as defined in claims 45, 46, 58 and 59. Kits and diagnostic compositions as defined in claims 47 and 48. A multispecific antibody obtained by the method as described in claims 58 or 59.

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2. claims: 49-51

   An antibody a defined in claim 49.

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3. claims: 52-57

   An antibody as defined in claim 52.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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