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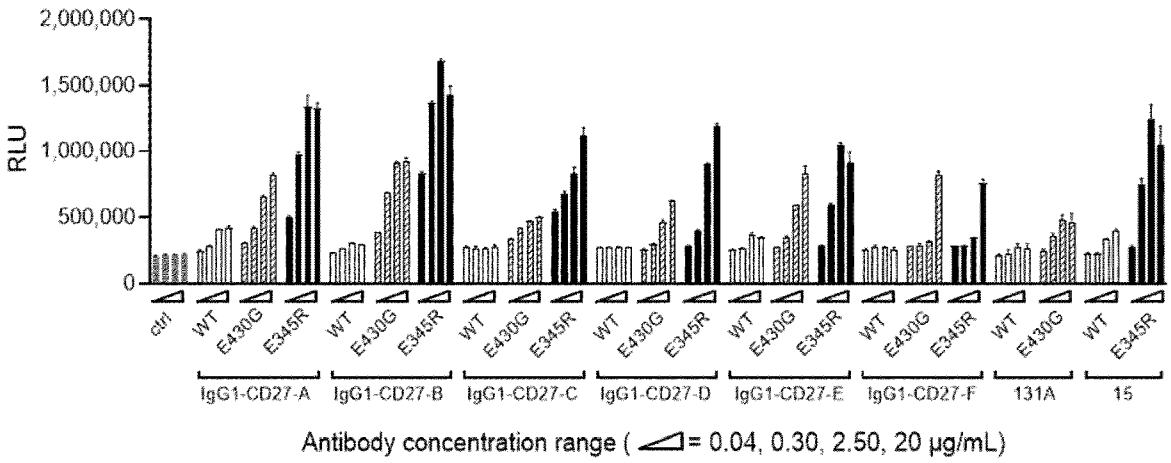
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(54) Title: BINDING AGENTS CAPABLE OF BINDING TO CD27 IN COMBINATION THERAPY

Figure 1




(57) Abstract: The present invention provides combination therapy using a first binding agent comprising at least one binding region binding to CD27 in combination with a second binding agent comprising a first binding region binding to CD40 and a second binding region binding to CD137 to reduce progression or prevent progression of a tumor or treating cancer.

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# BINDING AGENTS CAPABLE OF BINDING TO CD27 IN COMBINATION THERAPY

## TECHNICAL FIELD

The present invention relates to combination therapy using a first binding agent comprising at least one binding region binding to CD27 in combination with a second binding agent comprising a first binding region binding to CD40 and a second binding region binding to CD137 to reduce progression or prevent progression of a tumor or treating cancer.

## BACKGROUND OF THE INVENTION

Cluster of differentiation (CD)27 (TNFRSF7) is a 55kDa type I transmembrane protein member of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) which co-stimulates T-cell activation after binding to its ligand CD70. It is expressed in humans on the cell membrane of T, B, natural killer (NK) cells, and their immediate precursors, all of them part of the lymphoid lineage. On human T cells, CD27 is expressed on resting αβ $CD4^+$ (Treg and conventional T cells), $CD8^+$ T cells, stem-cell memory cells, and central-memory-like cells. On human B cells, CD27 is a memory B cell marker and CD27 signaling promotes differentiation of B cells into plasma cells.

The only known ligand for CD27 is the type II transmembrane protein CD70 (tumor necrosis factor superfamily member 7, TNFSF7; CD27 ligand, CD27L), which is quite restrictively and only transiently expressed on activated immune cells, including T, B, NK, and dendritic cells (DCs).

CD27 plays a role in early generation of a primary immune response and is required for generation and long-term maintenance of T-cell immunity. CD27-CD70 binding leads to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK)8/Jun N-terminal kinase (JNK) pathways. Adaptor proteins TNF receptor-associated protein (TRAF)2 and TRAF5 have been shown to mediate the signaling resulting from CD27 engagement.

To unlock their effector functions, T cells require T-cell antigen receptor-mediated recognition of their cognate antigen in the context of major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs), and activation of costimulatory receptors. CD27 and CD28 are considered the most important costimulatory receptors expressed on T cells.

In mice, CD27 stimulation during the priming phase of T-cell activation, has been found to promote clonal expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells by interleukin (IL)-2-independent survival signaling (Carr JM et al, Proc Natl Acad Sci USA 2006 Dec 19; 130(51):19454-9). CD27 also counteracts apoptosis of activated T cells throughout successive divisions and was also shown to play an important role in memory differentiation of mouse $CD8^+$ T cells. (Van de Ven K, Borst J. Immunotherapy 2015;7(6):655-67). As a result, CD27 stimulation promotes the generation of effector T cells in lymphoid organs and broadens the responder T-cell repertoire. In human naïve T cells, CD27 stimulation promotes T helper-1 (Th1) differentiation of $CD4^+$ T cells and supports effector differentiation of cytotoxic T-lymphocytes (Oosterwijk et al, Int Immunol. 2007 Jun; 19(6):713-8).

Contrarily to its presence on tumor cells in some hematological malignancies, CD27 expression has not been detected on tumor cells in solid malignancies. However, CD27-expressing lymphoid cells have been described in the tumor microenvironment (TME) of both hematological malignancies and solid cancers.

In the treatment of cancer, engagement and stimulation of the immune response has been shown to induce and/or enhance anti-tumor immunity resulting in clinical responses, as exemplified by the clinical success of immune checkpoint inhibitors (CPIs). An active immune response and/or existing anti-tumor immunity can be increased by providing costimulatory signaling, for example CD27 costimulatory signaling.

In mouse tumor models, T-cell functions and therefore antitumor immunity can be enhanced by agonistic CD27 antibodies. In human CD27 (hCD27)-transgenic lymphoma mouse models, CD27 activation using agonistic antibodies showed potent antitumor activity and induction of protective immunity, which is dependent on $CD4^+$ and $CD8^+$ T cells (He LZ et al., J Immunol. 2013 Oct 15;191(8):4174-83). Furthermore, CD27 activation using monoclonal antibodies prevented tumor growth in mouse xenografts, including models derived from leukemia (Vitale et al, Keler T. Clin Cancer Res. 2012 Jul 15;18(14):3812-21), melanoma (Roberts DJ, et al., J Immunother. 2010 Oct;33(8):769-79), colon carcinoma, and thymoma (He LZ, et al., J Immunol. 2013 Oct 15;191(8):4174-83), among others.

Monoclonal immunoglobulin G (IgG)1 agonistic antibodies against human CD27 have been disclosed in the prior art.

In WO2012/004367 a humanized anti-human CD27 agonistic antibody (designated hCD27.15) is described. It is reported that hCD27.15 does not require crosslinking by fragment crystallizable (Fc) gamma receptor (FcγR)-expressing cells to activate CD27-mediated costimulation of the immune response. However, this antibody does not bind to a

frequently occurring single nucleotide polymorphism (SNP) in hCD27 (A59T) and does not bind to cynomolgus monkey CD27.

WO2011/130434 discloses a human agonistic anti-human CD27 antibody designated 1F5, which activates CD27 upon crosslinking by FcyR-expressing cells and further blocks the binding of soluble CD70 (sCD70) ligand binding. 1F5 is reported to have Fc-mediated effector function activity, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) on target cells and to enhance the immune response and to have anti-tumor activity in mouse models.

WO2018/058022 discloses the agonistic murine anti-human CD27 antibody 131A and humanized versions thereof. It is disclosed that 131A binds the frequently occurring hCD27 SNP A59T and to cynomolgus monkey CD27. WO2018/058022 further discloses that in a mouse tumor model, antibody 131A had greater antitumor response compared with the antibody 1F5.

WO2019/195452 discloses the non-ligand blocking agonistic anti-human CD27 antibody designated BMS-986215, which is reported to have a higher affinity for human and cynomolgus monkey CD27 than the CD27 antibody 1F5 mentioned above. It is disclosed that in the presence of BMS-986215, CD27 costimulation of T cells occurs by binding to its ligand CD70. It is further disclosed that BMS-986215 reduces the suppression of $CD4^+$ responder T cells by regulatory T cells (Tregs) and that BMS-986215 binds C1q and induces CDC, modest ADCC and low levels of antibody-dependent cellular phagocytosis (ADCP). It is further disclosed that BMS-986215 only has weak agonist activity in the absence of FcyR and in the absence of sCD70.

Anti-CD27 antibodies must induce clustering of CD27 on the plasma membrane to induce CD27 agonism. In the case of wild type IgG1 antibodies, clustering of CD27 may be achieved through interaction of membrane-bound CD27 antibodies with FcyR-bearing cells, such as monocytes, macrophages, B cells and other immune cells. As a consequence, anti-CD27 IgG1 molecules may be less efficient when the number of FcyR-expressing cells is limited. Optimization of the effector functions by modifications of the Fc region of the antibody may improve the effectivity of therapeutic antibodies for treating cancer or other diseases, e.g., to improve the ability of an antibody to elicit an immune response to antigen-expressing cells. Such efforts are described in, e.g., WO 2013/004842 A2; WO 2014/108198 A1; WO2018/146317; WO2018/083126; WO 2018/031258 A1; Dall'Acqua, Cook et al. J Immunol 2006, 177(2): 1129-1138; Moore, Chen et al. MAbs 2010 2(2): 181-189; Desjarlais and Lazar, Exp Cell Res 2011, 317(9): 1278-1285; Kaneko and Niwa, BioDrugs 2011, 25(1): 1-

11; Song, Myojo et al., Antiviral Res 2014, 111: 60-68; Brezski and Georgiou, Curr Opin Immunol 2016, 40: 62-69; Sondermann and Szymkowski, Curr Opin Immunol 2016, 40: 78-87; Zhang, Armstrong et al. MAbs 2017, 9(7): 1129-1142.; Wang, Mathieu et al. Protein & Cell 2018, 9(1): 63-73; Diebolder FJ et al., Science. 2014 Mar 14;343(6176):1260-3).

Amongst others, Garber et al discussed opportunities for combination therapies consisting of agonistic antibodies targeting costimulatory receptors on T cells, e.g., 4-1BB (CD137), OX40, glucocorticoid-induced tumor necrosis factor receptor family-related receptor (GITR) and independent co-stimulation (ICOS), and monoclonal antibodies blocking the PD-1/PD-L1 axis (Garber et al. Nat Rev Drug Discov. 2020 Jan;19(1):3-5). Azpilikueta et al. (J Thorac Oncol 2016;11:524–36) have published preclinical data from a combination therapy comprising a PD-1-blocking antibody and a 4-1BB-targeting antibody in a mouse lung carcinoma model, showing that the combination therapy outperformed single-agent treatment. Also, Diggs et al published on the improved antitumor activity by a combination therapy of a PD-1-blocking antibody with an anti-CD40 antibody in a murine tumor hepatic carcinoma model (Diggs et al. J Hepatol. 2021 May;74(5):1145-1154).

WO2008/051424A2 provides methods comprising the administration of a CD27-targeting agonistic antibody alone, or combined with other immunomodulatory agents, such as antibodies targeting CD40, OX40, 4-1BB or CTLA-4.

Despite these and other efforts in the art, however, there is a need for improved antibody-based immunotherapies with increased agonism and/or increased potency to engage CD27, provided together as a combination therapy with other immunomodulatory antibodies.

## SUMMARY OF THE INVENTION

The present invention concerns binding agent capable of binding to CD27 in combination therapy.

In a first aspect, the present disclosure provides a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject i) a first binding agent comprises at least one binding region binding to CD27; and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In a second aspect, the present disclosure provides a kit comprising i) a first binding agent comprising at least one binding region binding to CD27 and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In a third aspect, the present disclosure provides a kit for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said kit comprising i) a first binding agent comprising at least one binding region binding to CD27 and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In a fourth aspect, the present disclosure provides a pharmaceutical composition comprising i) a first binding agent comprising at least one binding region binding to CD27; ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137; and iii) optionally a pharmaceutical acceptable carrier.

In a fifth aspect, the present disclosure provides a pharmaceutical composition for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said pharmaceutical composition comprising i) a first binding agent comprising at least one binding region binding to CD27, ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137; and iii) optionally a pharmaceutical acceptable carrier.

In a sixth aspect, the present disclosure provides a first binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject i) the first binding agent comprising at least one binding region binding to CD27; and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In a seventh aspect, the present disclosure provides a second binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject i) a first binding agent comprising at least one binding region binding to CD27; and ii) the second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

## DETAILED DESCRIPTION OF THE INVENTION

### *Definitions*

The term "antibody" (Ab) in the context of the present invention refers 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. The antibody of the present invention comprises an Fc-domain of an immunoglobulin and an antigen-binding region. An antibody generally contains two CH2-CH3 regions and a connecting region, e.g., a hinge region, e.g. at least an Fc-domain. Thus, the antibody of the present invention may comprise an Fc region and an antigen-binding region. The variable regions of the heavy and light chains of the immunoglobulin molecule contain a binding domain that interacts with an antigen. The constant or "Fc" regions of the antibodies 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. As used herein, unless contradicted by context, the Fc region of an immunoglobulin typically contains at least a CH2 domain and a CH3 domain of an immunoglobulin CH, and may comprise a connecting region, e.g., a hinge region. An Fc-region is typically in dimerized form via, e.g., disulfide bridges connecting the two hinge regions and/or non-covalent interactions between the two CH3 regions. The dimer may be a homodimer (where the two Fc region monomer amino acid sequences are identical) or a heterodimer (where the two Fc region monomer amino acid sequences differ in one or more amino acids). An Fc region-fragment of a full-length antibody can, for example, be generated by digestion of the full-length antibody with papain, as is well-known in the art. An antibody as defined herein may, in addition to an Fc region and an antigen-binding region, further comprise one or both of an immunoglobulin CH1 region and a CL region. An antibody may also be a multi-specific antibody, such as a bispecific antibody or similar molecule. The term "bispecific antibody" refers to an antibody having specificities for at least two different, typically non-overlapping, epitopes. Such epitopes may be on the same or different targets. If the epitopes are on different targets, such targets may be on the same cell or different cells or cell types. As indicated above, unless otherwise stated or clearly contradicted by the context, the term antibody herein includes fragments of an antibody which comprise at least a portion of an Fc-region and which retain the ability to specifically bind to the antigen. Such fragments may be provided by any known technique, such as enzymatic cleavage, peptide synthesis and recombinant expression techniques. 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 "Ab" or "antibody" include, without limitation, monovalent antibodies (described in WO2007059782 by Genmab); heavy-chain antibodies, consisting only of two heavy chains and naturally occurring in e.g. camelids (e.g., Hamers-Casterman (1993) Nature 363:446); ThioMabs, Roche, WO2011069104); strand-exchange engineered domain (SEED or Seed-body) which are asymmetric and bispecific antibody-like molecules (Merck, WO2007110205); Triomab (Pharma/Fresenius Biotech, Lindhofer et al. 1995 J Immunol 155:219; WO2002020039); FcΔAdp (Regeneron, WO2010151792); Azymetric Scaffold (Zymeworks/Merck, WO2012/058768); mAb-Fv (Xencor, WO2011/028952); Xmab (Xencor); Dual variable domain immunoglobulin (Abbott, DVD-Ig,U.S. Patent No. 7,612,181); Dual domain double head antibodies (Unilever; Sanofi Aventis, WO20100226923); Di-diabody (ImClone/Eli Lilly); Knobs-into-holes antibody formats (Genentech, WO9850431 ); DuoBody (Genmab, WO 2011/131746); Bispecific IgG1 and IgG2 (Pfizer/Rinat, WO11143545); DuetMab (MedImmune, US2014/0348839); Electrostatic steering antibody formats (Amgen, EP1870459 and WO 2009089004; Chugai, US201000155133; Oncomed, WO2010129304A2); bispecific IgG1 and IgG2 (Rinat neurosciences Corporation, WO11143545); CrossMAbs (Roche, WO2011117329); LUZ-Y (Genentech); Biclonic (Merus, WO2013157953); Dual Targeting domain antibodies (GSK/Domantis); Two-in-one Antibodies or Dual action Fabs recognizing two targets (Genentech, NovImmune, Adimab); Cross-linked Mabs (Karmanos Cancer Center); covalently fused mAbs (AIMM); CovX-body (CovX/Pfizer); FynomAbs (Covagen/Janssen ilag); DutaMab (Dutalys/Roche); iMab (MedImmune); IgG-like Bispecific (ImClone/Eli Lilly, Shen, J., et al. J Immunol Methods, 2007. 318(1-2): p. 65-74); TIG-body, DIG-body and PIG-body (Pharmabcine); Dual-affinity retargeting molecules (Fc-DART or Ig-DART, Macrogenics, WO/2008/157379, WO/2010/080538); BEAT (Glenmark); Zybodies (Zyngenia); approaches with common light chain (Crucell/ Merus, US7262028) or common heavy chains (κλBodies by NovImmune, WO2012023053), as well as fusion proteins comprising a polypeptide sequence fused to an antibody fragment containing an Fc-region like scFv-fusions, like BsAb by ZymoGenetics/BMS, HERCULES by Biogen Idec (US007951918); SCORPIONS (Emergent BioSolutions/Trubion and Zymogenetics/BMS); Ts2Ab (MedImmune/AZ (Dimasi, N., et al. J Mol Biol, 2009. 393(3): p. 672-92); scFv fusion (Genentech/Roche); scFv fusion (Novartis); scFv fusion (Immunomedics); scFv fusion (Changzhou Adam Biotech Inc, CN 102250246); TvAb (Roche,WO 2012025525, WO 2012025530); mAb2 (f-Star, WO2008/003116); and dual scFv-fusion. It should be understood that the term antibody, unless otherwise specified, includes monoclonal antibodies (such as human monoclonal antibodies), polyclonal

antibodies, chimeric antibodies, humanized antibodies, monospecific antibodies (such as bivalent monospecific antibodies), bispecific antibodies, antibodies of any isotype and/or allotype; antibody mixtures (recombinant polyclonals) for instance generated by technologies exploited by Symphogen and Merus (Oligoclonics), multimeric Fc proteins as described in WO2015/158867, and fusion proteins as described in WO2014/031646. While these different antibody fragments and formats 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.

An "agonistic antibody" for a natural receptor is a compound which binds the receptor to form a receptor-antibody complex and which activates said receptor, thereby initiating a pathway signaling and further biological process.

The term "agonism" and "agonistic" are used interchangeably herein and refer to or describe an antibody which is capable of, directly or indirectly, substantially inducing, promoting, or enhancing CD27 biological activity or activation. Optionally, an "agonistic CD27 antibody" is an antibody which is capable of activating CD27 receptor by a similar mechanism as the ligand for CD27, known as CD70 (Tumor Necrosis Factor Superfamily member 7, TNFSF7; CD27 ligand, CD27L), which results in an activation of one or more intracellular signaling pathway which may include activation of NF-KB and MAPK8/JNK pathways. "Agonism" as defined herein may be determined according to Example 2 herein.

A "CD27 antibody" or "anti-CD27 antibody" as described herein is an antibody which binds specifically to the protein CD27, in particular to human CD27.

A "variant" as used herein refers to a protein or polypeptide sequence which differs in one or more amino acid residues from a parent or reference sequence. A variant may, for example, have a sequence identity of at least 80%, such as 90%, or 95%, or 97%, or 98%, or 99%, to a parent or reference sequence. Also, or alternatively, a variant may differ from the parent or reference sequence by 12 or less, such as 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 mutation(s) such as substitutions, insertions, or deletions of amino acid residues. Accordingly, a "variant antibody" or an "antibody variant", used interchangeably herein, refers to an antibody that differs in one or more amino acid residues as compared to a parent or reference antibody, e.g., in the antigen-binding region, Fc-region or both. Likewise, a "variant Fc region" or "Fc region variant" refers to an Fc region that differs in one or more amino acid residues as compared to a parent or reference Fc region, optionally differing from the parent or reference Fc region amino acid sequence by 12 or less, such as 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 mutation(s) such as substitutions, insertions, or deletions of amino acid residues. The parent

or reference Fc region is typically the Fc region of a human wild-type antibody which, depending on the context, may be a particular isotype. A variant Fc region may, in dimerized form, be a homodimer or heterodimer, e.g., where one of the amino acid sequences of the dimerized Fc region comprises a mutation while the other is identical to a parent or reference wild-type amino acid sequence. Examples of wild-type (typically a parent or reference sequence) IgG CH and variant IgG constant region amino acid sequences, which comprise Fc region amino acid sequences, are set out in Table 3.

The term "immunoglobulin heavy chain" or "heavy chain of an immunoglobulin" as used herein is intended to refer to one of the heavy chains of an immunoglobulin. A heavy chain is typically comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region (abbreviated herein as CH) which defines the isotype of the immunoglobulin. The heavy chain constant region typically is comprised of three domains, CH1, CH2, and CH3. The term "immunoglobulin" as used herein is intended to refer to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains and one pair of heavy (H) chains, all four potentially inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized (see for instance Fundamental Immunology Ch. 7 Paul, W., 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. CDR sequences herein are defined according to IMGT (see Lefranc MP. et al., Nucleic Acids Research, 27, 209-212, 1999] and Brochet X. Nucl. Acids Res. 36, W503-508 (2008)).

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 below, including for example recombining by "half-molecule exchange" also described in the art as "Fab-arm exchange" and the DuoBody® method, as well as recombining at nucleic acid level and/or through co-expression of two half-molecules in the same cells.

The term "antigen-binding region" or "binding region" or antigen-binding domain as used herein, refers to the region of an antibody which is capable of binding to the antigen. This binding region is typically defined by the VH and VL domains of the antibody which 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). The antigen can be any molecule, such as a polypeptide, e.g., present on a cell, bacterium, or virion. The terms "antigen-binding region" and "antigen-binding site" and "antigen-binding domain" may, unless contradicted by the context, be used interchangeably in the context of the present invention.

The terms "antigen" and "target" may, unless contradicted by the context, be used interchangeably in the context of the present invention.

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

The term "$K_D$" (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction, and is obtained by dividing $k_d$ by $k_a$.

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 "CD27" as used herein, refers to the human protein entitled CD27, also known as tumor necrosis factor receptor superfamily member 7 (TNFRSF7). In the amino acid sequence shown in SEQ ID NO: 1 (Uniprot ID P26842), amino acid residues 1-19 are a signal peptide, and amino acid residues 20-240 are the mature polypeptide. Unless contradicted by context, CD27 may also refer to variants of CD27, isoforms and orthologs thereof. A naturally occurring variant of human CD27 comprising a A59T mutation is shown in SEQ ID NO: 2.

In cynomolgus monkey (Macaca fascicularis), the CD27 protein has the amino acid sequence shown in SEQ ID NO: 3 (Genbank XP_005569963). In the 240 amino acid sequence shown in SEQ ID NO: 3, the signal peptide is not defined.

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 binding using biolayer interferometry, by alanine scan, or by 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 by crystallography of the antibody bound to its antigen.

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

The terms "monoclonal antibody", "monoclonal Ab", "monoclonal antibody composition", "mAb", or the like, as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. 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 non-human animal, such as a transgenic mouse or rat, having a genome comprising a human heavy chain transgene and a light chain transgene, fused to an immortalized cell. 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 IgG, 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 (☐) or lambda (☐) light chain.

The term "full-length antibody" when used herein, indicates that the antibody is not a fragment, but contains all of the domains of the particular isotype normally found for that isotype in nature, e.g., the VH, CH1, CH2, CH3, hinge, VL and CL domains for an IgG1 antibody. In a full-length variant antibody, the heavy and light chain constant and variable domains may in particular contain amino acid substitutions that improve the 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 a suitable vector comprising complete heavy chain sequences and complete light chain sequence, and (ii) expressing the complete 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 would know how to generate a full-length antibody according to the present invention.

The term "human antibody", as used herein, is intended to include antibodies comprising variable and framework regions derived from human germline immunoglobulin sequences and a human immunoglobulin constant domain. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations, insertions or deletions introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another non-human species, such as a mouse, have been grafted onto human framework sequences.

The term "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 the six non-human antibody complementarity-determining regions

(CDRs), which together form the antigen binding site, onto a homologous human acceptor framework region (FR) (see WO92/22653 and EP0629240). In order to fully reconstitute the binding affinity and specificity of the parental antibody, the substitution of framework residues from the parental antibody (i.e., the non-human antibody) into the human framework regions (back-mutations) may be required. Structural homology modeling may help to identify the amino acid residues in the framework regions that are important for the binding properties of the antibody. Thus, a humanized antibody may comprise non-human CDR sequences, primarily human framework regions optionally comprising one or more amino acid back-mutations to the non-human amino acid sequence, and fully human constant regions. Optionally, additional amino acid modifications, which are not necessarily back-mutations, may be applied to obtain a humanized antibody with preferred characteristics, such as affinity and biochemical properties.

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

The term "parent polypeptide" or "parent antibody", is to be understood as a polypeptide or antibody, which is identical to a polypeptide or antibody according to the invention, but where the parent polypeptide or parent antibody is without mutations, unless otherwise stated or clearly contradicted by the context. For example, the antibody IgG1-CD27-A of the invention is the parent antibody of IgG1-CD27-A-P329R-E345R.

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 (Eu-index) 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" or "Fc effector functions" as used herein are used interchangeably and 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 cytotoxity (ADCC), (v) Fc-gamma receptor (FcγR)-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 "decreased Fc effector function(s)" or "Decreased Fc-mediated effector functions", as used herein are used interchangeably and is intended to refer to an Fc effector function that is decreased for an antibody when directly compared to the Fc effector function of the parent polypeptide or antibody in the same assay.

The term "inertness", "inert" or "non-activating" as used herein, refers to an Fc region which is at least not able to bind any FcγR, induce Fc-mediated cross-linking of FcγRs, or induce FcγR-mediated cross-linking of target antigens via two Fc regions of individual antibodies, or is not able to bind C1q. Thus, in certain embodiments of the invention the Fc region is inert. Therefore, in certain embodiments some or all of the Fc-mediated effector functions are attenuated or completely absent.

The term "oligomerization", as used herein, is intended to refer to a process that converts monomers to a finite degree of polymerization. Antibodies according to the invention can form oligomers, such as hexamers, via non-covalent association of Fc-regions after target binding, e.g., at a cell surface. Oligomerization of anti-CD27 antibodies upon cell surface binding through Fc:Fc interactions may increase CD27 clustering resulting in activation of CD27

intracellular signaling. The capacity of antibodies comprising the E345R or E430G mutation to form oligomers, such as hexamers, upon cell surface binding can be evaluated as described in: de Jong RN et al, PLoS Biol. 2016 Jan 6;14(1):e1002344. Fc-Fc-mediated oligomerization of antibodies occurs after target binding on a (cell) surface through the intermolecular association of Fc-regions between neighboring antibodies and is increased by introduction of a E345R or a E430G mutation (numbering according to Eu-index).

The term "clustering", as used herein, refers to oligomerization of antibodies through non-covalent interactions.

The term "Fc-Fc enhancing", as used herein, is intended to refer to increasing the binding strength between, or stabilizing the interaction between, the Fc regions of two Fc-region containing antibodies so that the antibodies form oligomers such as hexamers on the cell surface. This enhancement can be obtained by certain amino acid mutations in the Fc regions of the antibodies, such as E345R or E430G. The term "monovalent antibody", in the context of the present invention, refers to an antibody molecule that can interact with a specific epitope on an antigen, with only one antigen binding domain (e.g. one Fab arm). In the context of a bispecific antibody, "monovalent antibody binding" refers to the binding of the bispecific antibody to one specific epitope on an 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 epitope only. The antibody may be a monospecific, monovalent antibody (i.e. carrying only one antigen binding region) or a monospecifc, bivalent antibody (i.e. an antibody with two identical antigen binding regions).

The term "bispecific antibody" refers to an antibody comprising two non-identical antigen binding domains, e.g. two non-identical Fab-arms or two Fab-arms with non-identical CDR regions. In the context of this invention, bispecific antibodies have specificity for at least two different epitopes. Such epitopes may be on the same or different antigens or targets. If the epitopes are on different antigens, such antigens may be on the same cell or different cells, cell types or structures, such as extracellular matrix or vesicles and soluble protein. A bispecific antibody may thus be capable of crosslinking multiple antigens, e.g. two different cells. A particular bispecific antibody of the present invention is capable of binding to CD27 and a second target.

The term "bivalent antibody" refers to an antibody that has two antigen binding regions, which bind to epitopes on one or two targets or antigens or binds to one or two epitopes on the

same antigen. Hence, a bivalent antibody may be a monospecific, bivalent antibody or a bispecific, bivalent 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 5. 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 6. Alternative Physical and Functional Classifications of Amino Acid Residues*

| Class | Amino acid |
|---|---|
| Hydroxyl group containing residues | S and T |
| Aliphatic residues | I, L, V, and M |
| Cycloalkenyl-associated residues | F, H, W, and Y |
| Hydrophobic residues | A, C, F, G, H, I, L, M, R, T, V, W, and Y |
| Negatively charged residues | D and E |
| Polar residues | C, D, E, H, K, N, Q, R, S, and T |
| Positively charged residues | H, K, and R |
| Small residues | A, C, D, G, N, P, S, T, and V |
| Very small residues | A, G, and S |
| Residues involved in turn formation | A, C, D, E, G, H, K, N, Q, R, S, P, and T |
| Flexible residues | Q, T, K, S, G, P, D, E, and R |

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

In the context of the present invention, a substitution in an antibody is indicated as:

Original amino acid – position – substituted amino acid;

Referring to the well-recognized nomenclature for amino acids, the three-letter code, or one letter code, is used, including the codes "Xaa" or "X" to indicate any amino acid residue. Thus, Xaa or X may typically represent any of the 20 naturally occurring amino acids. The term "naturally occurring" as 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. This is, by the way, equivalent to the

designation 409X, wherein the X designates any amino acid other than the original amino acid. These substitutions may also be designated K409A, K409C, etc. or K409A,C, etc. or K409A/C/etc. The same applies by analogy to each and every position mentioned herein, to specifically include herein any one of such substitutions.

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

The term "host cell", as used herein, is intended to refer to a cell into which 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 also to 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 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, the sequence identity between two amino acid sequences is determined 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 sequence.

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

As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, for instance lymphocytes (such as B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, polymorphonuclear cells, such as neutrophils, granulocytes, mast cells, and basophils. Some effector cells express Fc receptors (FcgRs) or complement receptors and carry out specific immune functions. In some embodiments, an effector cell such as, e.g., a natural killer cell, is capable of inducing ADCC. For example, monocytes, macrophages, neutrophils, dendritic cells and Kupffer cells which express FcgRs, are involved in specific killing of target cells and/or presenting antigens to other components of the immune system, or binding to cells that present antigens. In some embodiments the ADCC can be further enhanced by antibody driven classical complement activation resulting in the deposition of activated C3 fragments on the target cell. C3 cleavage products are ligands for complement receptors (CRs), such as CR3, expressed on myeloid cells. The recognition of complement fragments by CRs on effector cells may promote enhanced Fc receptor-mediated ADCC. In some embodiments antibody driven classical complement activation leads to C3 fragments on the target cell. These C3 cleavage products may promote direct complement-dependent cellular cytotoxicity (CDCC). In some embodiments, an effector cell may phagocytose a target antigen, target particle or target cell which may depend on antibody binding and mediated by FcγRs expressed by the effector cells. The expression of a particular FcR or complement receptor on an effector cell may be regulated by humoral factors such as cytokines. For example, expression of FcγRI has been found to be up-regulated by interferon γ (IFN γ) and/or G-CSF. This enhanced expression increases the cytotoxic activity of FcγRI-bearing cells against targets. An effector cell can phagocytose a target antigen or phagocytose or lyse a target cell. In some embodiments antibody driven classical complement activation leads to C3 fragments on the target cell. These C3 cleavage products may promote direct phagocytosis by effector cells or indirectly by enhancing antibody mediated phagocytosis. In certain embodiments herein where the antibody has an inert Fc region the antibody does not induce an Fc-mediated effector function.

"Effector T cells" or "Teffs" or "Teff" as used herein refers to T lymphocytes that carry out a function of an immune response, such as killing tumor cells and/or activating an antitumor immune-response which can result in clearance of the tumor cells from the body. Examples of Teff phenotypes include $CD3^+CD4^+$ and $CD3^+CD8^+$. Teffs may secrete, contain, or express markers such as IFNγ, granzyme B and ICOS. It is appreciated that Teffs may not be fully restricted to these phenotypes.

"Memory T cells" as used herein refers to T lymphocytes that remain in the body for a long period of time after an infection is removed. Examples of memory T cells include central memory T cells (CD45RA-CCR7+) and effector memory T cells (CD45RA-CCR7-). It is appreciated that memory T cells may not be fully restricted to these phenotypes.

"Regulatory T cells" or "'Tregs" or "Treg" as used herein refers to T lymphocytes that regulate the activity of other T cell(s) and/or other immune cells, usually by suppressing their activity. An example of a Treg phenotype is $CD3^+CD4^+CD25^+CD127dim$. Tregs may further express Foxp3. It is appreciated that Tregs may not be fully restricted to this phenotype.

As used herein, the term "complement activation" refers to the activation of the classical complement pathway, which is initiated by a large macromolecular complex called C1 binding to antibody-antigen complexes on a surface. C1 is a complex, which consists of 6 recognition proteins C1q and a hetero-tetramer of serine proteases, C1r2C1s2. C1 is the first protein complex in the early events of the classical complement cascade that involves a series of cleavage reactions that starts with the cleavage of C4 into C4a and C4b and C2 into C2a and C2b. C4b is deposited and forms together with C2a an enzymatic active convertase called C3 convertase, which cleaves complement component C3 into C3b and C3a, which forms a C5 convertase This C5 convertase splits C5 in C5a and C5b and the last component is deposited on the membrane and that in turn triggers the late events of complement activation in which terminal complement components C5b, C6, C7, C8 and C9 assemble into the membrane attack complex (MAC). The complement cascade results in the creation of pores in the cell membrane which causes lysis of the cell, also known as complement-dependent cytotoxicity (CDC). In certain embodiments herein where the antibody has an inert Fc region the antibody does not induce complement activation.

Complement activation can be evaluated by using C1q binding efficacy, CDC kinetics CDC assays (as described in WO2013/004842, WO2014/108198) or by the method Cellular deposition of C3b and C4b described in Beurskens et al., J Immunol April 1, 2012 vol. 188 no. 7, 3532-3541.

The term "C1q binding" as used herein, is intended to refer to the binding of C1q in the context of the binding of C1q to an antibody bound to its antigen. The antibody bound to its antigen is to be understood as happening both *in vivo* and *in vitro* in the context described herein. C1q binding can be evaluated for example by using antibody immobilized on artificial surfaces or by using antibody bound to a predetermined antigen on a cellular or virion surface, as described in Example 8 herein. The binding of C1q to an antibody oligomer is to be understood herein as a multivalent interaction resulting in high avidity binding. A decrease in C1q binding, for example resulting from the introduction of a mutation in the antibody of the invention, may be measured by comparing the C1q binding of the mutated antibody to the C1q binding of its parent antibody (the antibody of the invention without the mutation within the same assay).

The term "treatment" refers to the administration of an effective amount of a therapeutically active antibody of the present invention with the purpose of easing, ameliorating, arresting, or eradicating (curing) symptoms or disease states.

The term "effective amount" or "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount of an antibody may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody variant are outweighed by the therapeutically beneficial effects.

The term "pharmacokinetic profile "as used herein can be determined as the plasma IgG levels over time as described in Example 12 herein.

The term "CD40" as used herein, refers to CD40, also referred to as tumor necrosis factor receptor superfamily member 5 (TNFRSF5), which is the receptor for the ligand TNFSF5/CD40L. CD40 is known to transduce TRAF6- and MAP3K8-mediated signals that activate ERK in macrophages and B cells, leading to induction of immunoglobulin secretion by the B cells. Other synonyms used for CD40 include, but are not limited to, B-cell surface antigen CD40, Bp50, CD40L receptor and CDw40. In one embodiment, CD40 is human CD40, having UniProt accession number P25942. The sequence of human CD40 is also shown in SEQ ID NO: 68. Amino acids 1-20 of SEQ ID NO: 68 correspond to the signal peptide of human CD40; while amino acids 21-193 of SEQ ID NO: 68 correspond to the extracellular domain of human CD40; and the remainder of the protein; i.e. from amino acids 194-215 and 216-277 of SEQ ID NO: 68 is transmembrane and cytoplasmic domain, respectively.

The term "CD137" as used herein, refers to CD137 (4-1BB), also referred to as tumor necrosis factor receptor superfamily member 9 (TNFRSF9), which is the receptor for the ligand TNFSF9/4-1BBL. CD137 (4-1BB) is believed to be involved in T-cell activation. Other synonyms for CD137 include, but are not limited to, 4-1BB ligand receptor, CDw137, T-cell antigen 4-1BB homolog and T-cell antigen ILA. In one embodiment, CD137 (4-1BB) is human CD137 (4-1BB), having UniProt accession number Q07011. The sequence of human CD137 is also shown in SEQ ID NO: 70. Amino acids 1-23 of SEQ ID NO: 70 correspond to the signal peptide of human CD137; while amino acids 24-186 of SEQ ID NO: 70 correspond to the extracellular domain of human CD137; and the remainder of the protein, i.e. from amino acids 187-213 and 214-255 of SEQ ID NO: 70 are transmembrane and cytoplasmic domain, respectively.

Fc regions may have at their C-terminus a lysine. The origin of this lysine is a naturally occurring sequence found in humans from which these Fc regions are derived. During cell culture production of recombinant antibodies, this terminal lysine can be cleaved off by proteolysis by endogenous carboxypeptidase(s), resulting in a constant region having the same sequence but lacking the C-terminal lysine. For manufacturing purposes of antibodies, the DNA encoding this terminal lysine can be omitted from the sequence such that antibodies are produced without the lysine. Antibodies produced from nucleic acid sequences that either do, or do not encode a terminal lysine are substantially identical in sequence and in function since the degree of processing of the terminal lysine is typically high when e.g. using antibodies produced in CHO-based production systems (Dick, L.W. et al. Biotechnol. Bioeng. 2008;100: 1132–1143). Hence, it is understood that proteins in accordance with the invention, such as antibodies, can be generated with or without encoding or having a terminal lysine. It is also understood in accordance with the invention that, sequences with a terminal lysine, such as a constant region sequence having a terminal lysine, can be understood as the corresponding sequences without a terminal lysine, and that sequences without a terminal lysine can also be understood as the corresponding sequences with a terminal lysine.

***Aspects and embodiments of the present disclosure***

In a first aspect, the present disclosure provides a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject i) a first binding agent comprises at least one binding region

binding to CD27; and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

*First binding agent binding to CD27*

In one embodiment, the first binding agent comprises at least one antigen-binding region capable of binding to human CD27 wherein said first binding agent comprises a heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and a light chain variable (VL) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10 and 11, respectively.

In a further embodiment , the first binding agent comprises two of said antigen-binding regions comprising the VH region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and the VL region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10 and 11 respectively. Hereby anti-CD27 antibodies are provided which are able to bind to human CD27 and further to bind to a variant of human CD27 comprising a mutation of A59T.

In an embodiment of the invention the first binding agent binds CD27 e.g. on T cells and is agonistic upon binding to its target. Hereby a first binding agent is provided which stimulates the activation and proliferation of T-cells. The first binding agent may further stimulate memory formation and survival of T-cells. Such a first binding agent is useful e.g. in the treatment of cancer. The first binding agent is further capable of binding to cynomolgus CD27 which is useful for toxicological studies of the first binding agent.

In one embodiment the first binding agent of the invention is an isolated antibody.

In one embodiment the first binding agent is an antibody. In another embodiment the first binding agent is a human antibody. In another embodiment the first binding agent is a humanized antibody. In another embodiment the first binding agent is a chimeric antibody.

The first binding agent of the invention is in a preferred embodiment a full-length antibody. Accordingly, the first binding agent of the invention may further comprise a light chain constant region (CL) and a heavy chain constant region (CH). The CH preferably comprises a CH1 region, a hinge region, a CH2 region and a CH3 region.

It is well known in the art that mutations in the VH and VL of an antibody can be made to, for example, increase the affinity of an antibody to its target antigen, reduce its potential immunogenicity and/or to increase the yield of antibodies expressed by a host cell. Accordingly, in some embodiments, first binding agents comprising variants of the CDR, VH and/or VL sequences of a first binding agent according to the invention are also contemplated, particularly functional variants of the VH and/or VL region as set forth in SEQ ID NO: 4 and SEQ ID NO: 8, respectively. Functional variants may differ in one or more amino acids as compared to the parent VH and/or VL sequence, e.g., in one or more CDRs, but still allows the antigen-binding region to retain at least a substantial proportion (at least about 50 percent, 60 percent, 70 percent, 80 percent, 90 percent, 95 percent or more) or even retain all of the affinity and/or specificity of the parent antibody. Typically, such functional variants retain significant sequence identity to the parent sequence. Exemplary variants include those which differ from the respective parent VH or VL region by 12 or less, such as 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 mutation(s) such as substitutions, insertions or deletions of amino acid residues. Exemplary variants include those which differ from the VH and/or VL and/or CDR regions of the parent sequences mainly by conservative amino acid substitutions; for instance, 12, such as 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 of the amino acid substitutions in the variant can be conservative. In a further embodiment of the invention the first binding agent may comprise at most 1, 2 or 3 mutations in the VH CDR region and/or in the VL CDR region, respectively. Such mutations may be substitutions. It is preferred that such substitutions do not significantly change the binding affinity and/or binding specificity of the first binding agent of the invention. Accordingly, the present invention encompasses variants of the first binding agent of the invention which variants have the same functional features as the first binding agent comprising the VH region CDR sequences as set forth in SEQ ID NOs: 5, 6, and 7, and the VL region CDR sequences as set forth in SEQ ID NO: 9, 10 and 11.

In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 80% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 85% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 90% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 95% identical to the VH region as set forth in SEQ ID

NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 96% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 97% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 98% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 99% identical to the VH region as set forth in SEQ ID NO: 4. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence as set forth in SEQ ID NO: 4.

In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 80% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 85% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 90% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 95% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 96% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 97% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 98% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence which is at least 99% identical to the VH region as set forth in SEQ ID NO: 8. In another embodiment of the invention the first binding agent comprises a VH region comprising a sequence as set forth in SEQ ID NO: 8.

In another embodiment of the invention the first binding agent comprises the VH and VL regions comprising the sequences as set forth in SEQ ID NO: 4 and SEQ ID NO: 8, respectively.

The first binding agent may comprise a light chain constant region which is a human kappa

light chain. In another embodiment it may comprise a human lambda light chain constant region.

The first binding agent according may preferably further comprise a heavy chain constant region, which is of a human IgG isotype. It may optionally comprise a modified human IgG constant region. Such human IgG comprise the Fc region which comprise the CH2 and CH3 region. By modifying the IgG constant region in the Fc region, it is for example possible to regulate the Fc effector functions of the antibody or to increase the Fc-Fc interactions and thereby the antibodies tendency to form clusters such as hexamers. In one embodiment of the invention the human IgG or modified human IgG is selected from IgG1, IgG2, IgG3 or IgG4. In one embodiment it is IgG1. In another embodiment it is IgG2. In yet another embodiment it is IgG3. In a further embodiment it is IgG4. In one particular embodiment the IgG is a modified human IgG comprising one or more amino acid substitutions in the Fc region. In one embodiment it may be a human IgG1 comprising one or more amino acid substitutions in the Fc region. In a further embodiment of the invention the IgG1 comprises two or more amino acid substitutions in the Fc region. In one embodiment the IgG1 Fc region has two amino acid substitutions.

In a further embodiment of the invention, the modified human IgG heavy chain constant region comprises in the Fc region at most 10 amino acid substitutions. In another embodiment it comprises at most 9 amino acid substitutions. In another embodiment it comprises at most 8 amino acid substitutions. In another embodiment it comprises at most 7 amino acid substitutions. In another embodiment it comprises at most 6 amino acid substitutions. In another embodiment it comprises at most 5 amino acid substitutions. In another embodiment it comprises at most 4 amino acid substitutions. In another embodiment it comprises at most 3 amino acid substitutions. In another embodiment it comprises at most 2 amino acid substitutions in the Fc region.

Mutations in amino acid residues at positions corresponding to E430, E345 and S440 in a human IgG1 heavy chain, wherein the amino acid residues are numbered according to the EU index, can improve the ability of an antibody to induce CDC. Without being bound by theory, it is believed that by substituting one or more amino acid(s) in these positions, oligomerization of the antibody can be stimulated, thereby modulating Fc-mediated effector functions so as to, e.g., increase C1q binding, complement activation, CDC, ADCP, internalization or other relevant function(s) that may provide in vivo efficacy.

In a further embodiment of the invention, the first binding agent is a variant antibody comprising an antigen-binding region and a variant Fc region.

In certain embodiments, an antibody variant binding to human CD27 comprises:
(a) a heavy chain comprising a VH region comprising a VH CDR1 comprising the sequence as set forth in SEQ ID NO:5, a VH CDR2 comprising the sequence as set forth in SEQ ID NO:6, a VH CDR3 comprising the sequence as set forth in SEQ ID NO:7 and a human IgG1 CH region comprising a mutation in one or more of E430, E345 and S440, the amino acid residues being numbered according to the EU index;
(b) a light chain comprising a VL region comprising a VL CDR1 comprising the sequence as set forth in SEQ ID NO:9, a VL CDR2 comprising the sequence as set forth in SEQ ID NO:10, and a VL CDR3 comprising the sequence as set forth in SEQ ID NO:11.

In other certain embodiments, an antibody variant binding to human CD27 comprises:
(a) a heavy chain comprising a VH region comprising SEQ ID NO:4 and a human IgG1 CH region comprising a mutation in one or more of E430, E345 and S440, the amino acid residues being numbered according to the EU index, and
(b) a light chain comprising a VL region comprising SEQ ID NO:8.

A variant antibody used according to the present invention comprises a variant Fc region or a variant human IgG1 CH region comprising a mutation in one or more of P329, E430 andE345. In the following, reference to the mutations in the Fc region may similarly apply to the mutation(s) in the human IgG1 CH region and vice versa.

As described herein, the position of an amino acid to be mutated in the Fc region can be given in relation to (i.e., "corresponding to") its position in a naturally occurring (wildtype) human IgG1 heavy chain, when numbered according to the Eu index. So, if the parent Fc region already contains one or more mutations and/or if the parent Fc region is, for example, an IgG2, IgG3 or IgG4 Fc region, the position of the amino acid corresponding to an amino acid residue such as, e.g., E430 in a human IgG1 heavy chain numbered according to the Eu index can be determined by alignment. Specifically, the parent Fc region is aligned with a wild-type human IgG1 heavy chain sequence so as to identify the residue in the position corresponding to E430 in the human IgG1 heavy chain sequence. Any wildtype human IgG1 constant region amino acid sequence can be useful for this purpose, including any one of the different human

IgG1 allotypes set forth in Table 3.

In one embodiment of the invention the modification in the IgG Fc region induces increased CD27 agonism compared to the identical antibody but comprising a wild type IgG Fc region of the same isotype, such as IgG1. This may for example be obtained by introducing an amino acid other than E at the amino acid position corresponding to position E345 and/or E430 in a human IgG1 heavy chain according to Eu numbering. In one embodiment of the invention the amino acid residue at the position corresponding to position E345 in a human IgG1 heavy chain according to Eu numbering is selected from the group comprising: A, C, D, F, G, H, I, K, L, M, N, Q, P, R, S, T, V, W and Y. In another embodiment of the invention the amino acid residue at the position corresponding to position E430 in a human IgG1 heavy chain according to Eu numbering is selected from the group comprising: A, C, D, F, G, H, I, K, L, M, N, Q, P, R, S, T, V, W.

In a preferred embodiment the amino acid residue at the position corresponding to position E345 in a human IgG1 heavy chain according to Eu numbering is R. Accordingly, the first binding agent of the invention may comprise an E345R substitution in the Fc region. In another embodiment of the invention the amino acid residue at the position corresponding to position E430 in a human IgG1 heavy chain according to Eu numbering is G. Accordingly, the first binding agent of the invention may comprise a E430G substitution in the Fc region. In another embodiment, the first binding agent comprises an amino acid substitution selected from the group comprising E430G, E345K, E430S, E430F, E430T, E345Q, E345R, E345Y.

Hereby, antibodies are provided which have enhanced Fc-Fc interaction which may lead to antibody-dependent clustering of CD27 on the cell surface upon antibody binding, thereby increasing the agonism of the antibody of the invention.

In another embodiment of the first binding agent of the invention the amino acid residue at the position corresponding to position P329 in a human IgG1 heavy chain according to Eu numbering is substituted with an amino acid selected from the group comprising: A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y. Accordingly, the first binding agent of the invention may further comprise a mutation in position 329.

In a further embodiment of the invention the first binding agent has the amino acid residue R at the position corresponding to position P329 in a human IgG1 heavy chain according to

Eu numbering. Accordingly, the first binding agent of the invention may have a P329R substitution in the Fc region. Without being bound by theory, it is believed that the first binding agent of the invention comprising an E345R mutation in the Fc region (as e.g. set out in SEQ ID NO: 13) has increased serum clearance. The inventors found that further introducing a mutation at position 329, such as P329R (as e.g. set out in SEQ ID NO: 15) restored the clearance of the first binding agent of the invention to the level of the first binding agent comprising a wt IgG1 as e.g. set out in SEQ ID NO: 12.

In another preferred embodiment the amino acid residues at the positions corresponding to positions P329 and E345 in a human IgG1 heavy chain according to Eu numbering are both R. Hereby a first binding agent is provided which has increased CD27 receptor agonism and comparable pharmacokinetic properties, such as e.g. serum clearance, when compared to a first binding agent comprising the same VH and VL region and comprising an identical IgG1 heavy chain constant region with the exception of comprising the wildtype amino acid P at position 329 and the wildtype amino acid E at position 345.

Thus, in an embodiment the invention provides a first binding agent which has increased receptor agonism upon binding to CD27 and which further has pharmacokinetic properties which are comparable, such as similar or even identical pharmacokinetic properties, when compared to the pharmacokinetic properties of a first binding agent comprising the same VH and VL region but comprising a wild type IgG1 heavy chain constant region such as e.g. set out in SEQ ID NO: 12. In other words the invention provides a first binding agent which has pharmacokinetic properties which are not significantly different than the pharmacokinetic properties of an identical first binding agent except for comprising a wild type IgG1 heavy chain constant region.

In other embodiments of the invention the first binding agent comprises a variant Fc region according to any one of the preceding sections, which variant Fc region is a variant of a human IgG Fc region selected from the group consisting of a human IgG1, IgG2, IgG3 and IgG4 Fc region. That is, the mutation in one or more of the amino acid residues corresponding to E430 and E345 and P329 is/are made in a parent Fc region which is a human IgG Fc region selected from the group consisting of an IgG1, IgG2, IgG3 and IgG4 Fc region. Preferably, the parent Fc region is a naturally occurring (wildtype) human IgG Fc region, such as a human wildtype IgG1, IgG2, IgG3 or IgG4 Fc region, or a mixed isotype thereof. Thus, the variant Fc region may, except for the recited mutation (in one or more of the amino acid residues selected from

E430 and E345 and P329), be a human IgG1, IgG2, IgG3 or IgG4 isotype, or a mixed isotype thereof.

In one embodiment, the parent Fc region and/or human IgG1 CH region is a wild-type human IgG1 isotype.

Thus, the variant Fc region may except for the recited mutation (in E430 or E345 or P329), be a human IgG1 Fc region.

In a specific embodiment, the parent Fc region and/or human IgG1 CH region is a human wild-type IgG1m(f) isotype.

In a specific embodiment, the parent Fc region and/or human IgG1 CH region is a human wild-type IgG1m(z) isotype.

In a specific embodiment, the parent Fc region and/or human IgG1 CH region is a human wild-type IgG1m(a) isotype.

In a specific embodiment, the parent Fc region and/or human IgG1 CH region is a human wild-type IgG1m(x) isotype.

In a specific embodiment, the parent Fc region and/or human IgG1 CH region is a human wild-type IgG1 of a mixed allotype, such as IgG1m(za), IgG1m(zax), IgG1m(fa), or the like.

Thus, the variant Fc region and/or human IgG1 CH region may, except for the recited mutation (in E430 or E345 or P329), be a human IgG1m(f), IgG1m(a), IgG1m(x), IgG1m(z) allotype or a mixed allotype of any two or more thereof.

In a specific embodiment, the parent Fc region and/or human IgG1 CH region is a human wild-type IgG1m(za) isotype.

In a specific embodiment, the parent Fc region is a human wild-type IgG2 isotype.

In a specific embodiment, the parent Fc region is a human wild-type IgG3 isotype.

In a specific embodiment, the parent Fc region is a human wild-type IgG4 isotype.

CH region amino acid sequences of specific examples of wild-type human IgG isotypes and IgG1 allotypes are set forth in Table 3.

In another embodiment the invention provides an first binding agent which comprises a heavy chain constant region comprising an amino acid sequence selected from the group comprising: SEQ ID Nos 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 27, 28, 29, 30, 31, 32, 33, 34 and 36. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 12. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 13. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 14. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 15. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 18. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 19. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 20. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 21. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 22. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 23. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 27. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 28. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 29. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 30. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 31. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 32. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 33. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 34. In one embodiment the heavy chain constant region has the amino acid sequence of SEQ ID NO: 36.

In an embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 15 and

d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 12 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 13 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 14 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 18 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 19 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8

c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 20 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 21 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 22 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 23 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 27 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 28 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4

b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 29 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 30 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 31 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 32 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 33 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:
a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 34 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In another embodiment the first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 36 and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

In alternative embodiments of the above first binding agents the CL region may be the amino acid sequence set forth in SEQ ID No: 17.

In an embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 15 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 12 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 13 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 14 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8

g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 18 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 19 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 20 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 21 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 22 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 23 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4

f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 27 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 28 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 29 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 30 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 31 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 32 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:

e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 33 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 34 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises:
e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4
f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8
g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 36 and
h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

In another embodiment the first binding agent comprises a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 24 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 25.

In another embodiment the first binding agent comprises a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 35 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 25.

In yet another embodiment the first binding agent comprises a heavy chain constant region that is modified so that the first binding agent induces an Fc-mediated effector function to a lesser extent relative to an identical first binding agent except for the modification. An example hereof is the CD27 binding antibody of the invention comprising a P329R and an E345R substitution. Such antibody induces one or more Fc-mediated effector function(s) to a lesser extent compared to the antibody comprising the same sequence except not comprising the P329R substitution and also compared to the same antibody comprising the same sequence except not comprising the P329R and E345R substitutions, such as a wildtype IgG1 heavy chain. In one embodiment the Fc-mediated effector function is decreased by at least 20%. In another embodiment the Fc-mediated effector function is decreased by at least 30%.

In another embodiment the Fc-mediated effector function is decreased by at least 40%. In another embodiment the Fc-mediated effector function is decreased by at least 50%. In another embodiment the Fc-mediated effector function is decreased by at least 60%. In another embodiment the Fc-mediated effector function is decreased by at least 70%. In another embodiment the Fc-mediated effector function is decreased by at least 80%. In another embodiment the Fc-mediated effector function is decreased by at least 90%. In another embodiment the first binding agent does not induce one or more Fc-mediated effector functions. The one or more Fc-effector functions that are decreased or not at all induced may be selected from the following group: complement-dependent cytotoxicity (CDC), complement-dependent cell-mediated cytotoxicity (CDCC), complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), C1q binding and FcγR binding. Accordingly, in one embodiment the first binding agent induces CDC to a degree which is decreased by at least 20%, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or decreased by at least 90% relative to the identical first binding agent but a wildtype IgG1 HC constant region. In another embodiment the first binding agent does not induce CDC.

In another embodiment, the first binding agent CDCC to a degree which is decreased by at least 20%, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or decreased by at least 90% relative to the identical first binding agent but having a wildtype IgG1 HC constant region. In another embodiment the first binding agent of the invention does not induce CDCC.

In another embodiment, the first binding agent ADCC to a degree which is decreased by at least 20%, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or decreased by at least 90% relative to the identical first binding agent but having a wildtype IgG1 HC constant region. In another embodiment the first binding agent does not induce ADCC.

In another embodiment, the first binding agent induces ADCP to a degree which is decreased by at least 20%, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or decreased by at least 90% relative to the identical first binding agent but having a wildtype IgG1 HC constant region. In another embodiment the first binding agent does not induce ADCP.

In another embodiment, the first binding agent induces C1q binding to a degree which is decreased by at least 20%, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or decreased by at least 90% relative to the identical first binding agent but having a wildtype IgG1 HC constant region. In another embodiment the first binding agent does not induce C1q binding. Preferably the C1q binding is determined as in example 8.

In another embodiment, the first binding agent induces FcγR binding to a degree which is decreased by at least 20%, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or decreased by at least 90% relative to the identical first binding agent but having a wildtype IgG1 HC constant region. In another embodiment the first binding agent of the invention does not induce FcγR binding. Preferably the FcγR binding is determined as in example 9.

In one embodiment the first binding agent has reduced C1q binding and reduced FcγR binding compared to the first binding agent comprising the same amino acid sequences except not comprising the P329R substitution.

In one embodiment, the first binding agent is, except for the recited mutations, a human antibody.

In an embodiment of the invention the first binding agent is a monovalent antibody.

In another embodiment the first binding agent is a bivalent antibody.

Further, the first binding agent may be a monospecific antibody.

In one embodiment, the first binding agent is a monoclonal antibody, such as a human monoclonal antibody, such as a human bivalent monoclonal antibody, such as a human bivalent full-length monoclonal antibody.

In a preferred embodiment, the first binding agent is, except for the optional recited mutations in the Fc region, an IgG1 antibody, such as a full length IgG1 antibody, such as a human full-length IgG1 antibody, optionally a human monoclonal full-length bivalent IgG1,κ antibody, e.g. a human monoclonal full-length bivalent IgG1m(f),κ antibody.

An first binding agent according to the present invention is advantageously in a bivalent monospecific format, comprising two antigen-binding regions binding to the same epitope. However, bispecific formats where one of the antigen-binding regions binds to a different epitope are also contemplated. So, the first binding agent according to any aspect or embodiment herein can, unless contradicted by context, be either a monospecific antibody or a bispecific antibody.

Accordingly, in another embodiment, the first binding agent is a bispecific antibody comprising a first antigen binding region capable of binding human CD27 as described herein and comprising a second antigen binding region capable of binding to a different epitope on human CD27. In another embodiment, the first binding agent is a bispecific antibody comprising a first antigen binding region capable of binding human CD27 as described herein and comprising a second antigen binding region capable of binding a different target. Such target may be on a different cell or on the same cell as CD27.

In an embodiment of the invention the first binding agent is capable of binding to human CD27 having the sequence as set forth in SEQ ID NO: 1. However, human CD27 may in some individuals be expressed as a variant hereof. Thus, in another embodiment the first binding agent of the invention is further capable of binding to a human CD27 variant, such as for example the human CD27 variant as set forth in SEQ ID NO: 2. In another embodiment, the first binding agent of the invention if further capable of binding to cynomolgus CD27, such as set forth in SEQ ID NO: 3.

In a further embodiment of the invention the first binding agent is capable of binding CD27-expressing human T cells.

In another embodiment of the invention the first binding agent is capable of binding CD27-expressing cynomolgus T cells.

In one embodiment of the invention the full length IgG1 antibody has had the C-terminal Lysine of the HC cleaved off. Such an antibody is also considered a "full length antibody".

In another embodiment of the invention the first binding agent is capable of inducing proliferation of human T cells such as $CD4^+$ and $CD8^+$ T-cells, such as T helper cells and

cytotoxic T cells. Such activity may be assayed as described in Example 6 or 7 herein.

In another embodiment of the invention the first binding agent is capable of inducing activation of human CD27-expressing Jurkat reporter T cells such as described in Example 2 herein.

In another embodiment of the invention the first binding agent is capable of inducing activation of human CD27-expressing Jurkat reporter T cells in the absence of Fcγ receptor IIb cross-linking such as described in Example 11 herein.

In another embodiment of the invention the first binding agent is capable of inducing proliferation of CD4+ and $CD8^+$ T cells with a central memory T cell phenotype.

In another embodiment of the invention the first binding agent is capable of inducing IFN gamma production.

In another embodiment of the invention the first binding agent is in a composition or formulation comprising acetate, sorbitol, polysorbate 80, and has a pH from 5 to 6, preferably 5.5.

*Second binding agent binding to CD40 and CD137*

In one embodiment, CD40 is human CD40, in particular human CD40 comprising the sequence set forth in SEQ ID NO: 62. In one embodiment, CD137 is human CD137, in particular human CD137 comprising the sequence set forth in SEQ ID NO: 63.

In one embodiment of the second binding agent, the second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In one embodiment of the second binding agent, a) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 44, 45, and 46, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 47, YTS , and SEQ ID NO: 48, respectively; and b) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1,

CDR2, and CDR3 sequences set forth in: SEQ ID NO: 51, 52, and 53, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 54, GAS, and SEQ ID NO: 55, respectively.

In one embodiment of the second binding agent, a) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 49 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 50;
and
b) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 56 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 57.

In one embodiment of the second binding agent, the second binding agent is a multispecific antibody, such as a bispecific antibody.

In one embodiment of the second binding agent, the second binding agent is in the format of a full-length antibody or an antibody fragment.

In one embodiment of the second binding agent, wherein the second binding agent is an antibody comprising a first binding arm and a second binding arm, wherein
the first binding arm comprises
i) a polypeptide comprising said first heavy chain variable region (VH) and a first heavy chain constant region (CH), and
ii) a polypeptide comprising said first light chain variable region (VL) and a first light chain constant region (CL);
and the second binding arm comprises
iii) a polypeptide comprising said second heavy chain variable region (VH) and a second heavy chain constant region (CH), and
iv) a polypeptide comprising said second light chain variable region (VL) and a second light chain constant region (CL).

In one embodiment of the second binding agent, said second binding agent comprises

i) a first heavy chain and light chain comprising said first binding region capable of binding to CD40, the first heavy chain comprising a first heavy chain constant region and the first light chain comprising a first light chain constant region; and
ii) a second heavy chain and light chain comprising said second binding region capable of binding CD137, the second heavy chain comprising a second heavy chain constant region and the second light chain comprising a second light chain constant region.

In one embodiment of the second binding agent, (i) the amino acid in the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering is L in said first heavy chain constant region (CH), and the amino acid in the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering is R in said second heavy chain constant region (CH), or (ii) the amino acid in the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering is R in said first heavy chain, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering is L in said second heavy chain.

In one embodiment of the second binding agent, the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to EU numbering are F and E, respectively, in said first and second heavy chains.

In one embodiment of the second binding agent, wherein the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain according to EU numbering are F, E, and A, respectively, in said first and second heavy chain constant regions (HCs).

In one embodiment of the second binding agent, the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to EU numbering of both the first and second heavy chain constant regions are F and E, respectively, and wherein (i) the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is L, and the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is R, or (ii) the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is R, and the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is L.

In one embodiment of the second binding agent, the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain according to EU numbering of both the first and second heavy chain constant regions are F, E, and A, respectively, and wherein (i) the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is L, and the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the second heavy chain constant region is R, or (ii) the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the first heavy chain is R, and the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is L.

In one embodiment of the second binding agent, the constant region of said first and/or second heavy chain, such as the second heavy chain, comprises or consists essentially of or consists of an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 58 or 60 [IgG1-Fc_FEAL];

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 6 substitutions, such as at most 5 substitutions, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

In one embodiment of the second binding agent, the constant region of said first and/or second heavy chain, such as the first heavy chain, comprises or consists essentially of or consists of an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 59 or 61 [IgG1-Fc_FEAR];

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 6 substitutions, such as at most 5 substitutions, at most 4, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

In one embodiment of the second binding agent, said second binding agent comprises a kappa (κ) light chain constant region.

In one embodiment of the second binding agent, said second binding agent comprises a lambda (λ) light chain constant region.

In one embodiment of the second binding agent, said first light chain constant region is a kappa (κ) light chain constant region or a lambda (λ) light chain constant region.

In one embodiment of the second binding agent, said second light chain constant region is a lambda (λ) light chain constant region or a kappa (κ) light chain constant region.

In one embodiment of the second binding agent, said first light chain constant region is a kappa (κ) light chain constant region and said second light chain constant region is a lambda (λ) light chain constant region or said first light chain constant region is a lambda (λ) light chain constant region and said second light chain constant region is a kappa (κ) light chain constant region.

In one embodiment of the second binding agent, the kappa (κ) light chain comprises an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 16,

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 10 substitutions, such as at most 9 substitutions, at most 8, at most 7, at most 6, at most 5, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

In one embodiment of the second binding agent according to the first aspect, the lambda (λ) light chain comprises an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 17,

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 10 substitutions, such as at most 9 substitutions, at most 8, at most 7, at most 6, at most 5, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

In one embodiment of the second binding agent according to the first aspect, the second binding agent is of an isotype selected from the group consisting of IgG1, IgG2, IgG3, and IgG4.

In one embodiment of the second binding agent according to the first aspect, the second binding agent is a full-length IgG1 antibody.

In one embodiment of the second binding agent according to the first aspect, the second binding agent is an antibody of the IgG1m(f) allotype.

In one embodiment, the second binding agent is a bispecific antibody binding to CD40 and CD137, the bispecific antibody having i) a first heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 64 and a first light chain comprising the amino acid sequence set forth in SEQ ID NO: 65 , and ii) a second heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 66 and a second light chain comprising the amino acid sequence set forth in SEQ ID NO: 67.

*Subject and tumor or cancer to be treated*

The subject to be treated according to the present disclosure is preferably a human subject.

In one embodiment the tumor or cancer is a solid tumor.

In one embodiment said tumor is a PD-L1 positive tumor.

In one embodiment the tumor or cancer is head and neck squamous cell carcinoma (HNSCC), such as HNSCC of the oral cavity, pharynx or larynx.

In one embodiment the HNSCC is recurrent, unresectable or metastatic.

In one embodiment the tumor or cancer is non-small cell lung cancer (NSCLC), such as a squamous or non-squamous NSCLC.

In one embodiment the NSCLC is recurrent, unresectable or metastatic.

In one embodiment the NSCLC does not have an epidermal growth factor (EGFR)-sensitizing mutation and/or anaplastic lymphoma (ALK) translocation and/or ROS1 rearrangement.

In one embodiment the NSCLC is NTRK1/2/3 (neurotrophic receptor tyrosine kinase 1/2/3) fusion positive, and/or has a mutation in KRAS (KRAS proto-oncogene, GTPase), BRAF (B-Raf proto-oncogene, serine/threonine kinase), or MET (MET proto-oncogene, receptor tyrosine kinase) gene, and/or has RET (ret proto-oncogene) gene rearrangements, and the subject has received prior treatment with a respective targeted therapy.

In one embodiment the subject has received prior treatment with a PD-1 inhibitor or a PD-L1 inhibitor, such as anti-PD-1 antibody or an anti-PD-L1 antibody, preferably at least two doses of the PD-1 inhibitor or the PD-L1 inhibitor.

In one embodiment the subject has received prior treatment with a platinum-based therapy or an alternative chemotherapy if platinum ineligible, e.g. a gemcitabine-containing regimen.

In one embodiment the tumor or cancer has relapsed and/or progressed after treatment, such as systemic treatment with a checkpoint inhibitor.

In one embodiment the subject has received at least one prior line of systemic therapy, such as systemic therapy comprising a PD-1 inhibitor or a PD-L1 inhibitor, such as an anti-PD-1 antibody or an anti-PD-L1 antibody.

In one embodiment the cancer or tumor has relapsed and/or is refractory, or the subject has progressed after treatment with a PD-1 inhibitor or a PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody, the PD-1 inhibitor or PD-L1 inhibitor being administered as monotherapy or as part of a combination therapy.

In one embodiment last prior treatment was with a PD1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody, the PD-1 inhibitor or PD-L1 inhibitor being administered as monotherapy or as part of a combination therapy.

In one embodiment the time from progression on last treatment with a PD-1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody is 6 months or less.

In one embodiment the time from last dosing of a PD-1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody as part of last prior treatment is 6 months or less.

In one embodiment the cancer or tumor has relapsed and/or is refractory, or the subject has progressed during or after i) platinum doublet chemotherapy following treatment with an anti-PD-1 antibody or an anti-PD-L1 antibody, or ii) treatment with an anti-PD-1 antibody or an anti-PD-L1 antibody following platinum doublet chemotherapy.

In a second aspect, the present disclosure provides a kit comprising i) a first binding agent comprising at least one binding region binding to CD27 and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In one embodiment of the kit according to the second aspect, the first binding agent is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the kit according to the second aspect, the second binding agent is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the kit according to the second aspect, the first binding agent, the second binding agent, and, if present, one or more additional therapeutic agents are for systemic administration, in particular for injection or infusion, such as intravenous injection or infusion.

In a third aspect, the present disclosure provides a kit for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said kit comprising i) a first binding agent comprising at least one binding region binding to CD27 and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In one embodiment of the kit for use according to the third aspect, the kit is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the kit for use according to the third aspect, the tumor or cancer is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the kit for use according to the third aspect, the subject is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the kit for use according to the third aspect, the method is as defined in any aspect or embodiment of the present disclosure.

In a fourth aspect, the present disclosure provides a pharmaceutical composition comprising i) a first binding agent comprising at least one binding region binding to CD27; ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137; and iii) optionally a pharmaceutical acceptable carrier.

In one embodiment of the pharmaceutical composition according to the fourth aspect, the first binding agent is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the pharmaceutical composition according to the fourth aspect, the second binding agent is as defined in any aspect or embodiment of the present disclosure.

In a fifth aspect, the present disclosure provides a pharmaceutical composition for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said pharmaceutical composition comprising i) a first binding agent comprising at least one binding region binding to CD27, ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137; and iii) optionally a pharmaceutical acceptable carrier.

In one embodiment of the pharmaceutical composition for use according to the fifth aspect, the pharmaceutical composition is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the pharmaceutical composition for use according to the fifth aspect, the tumor or cancer is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the pharmaceutical composition for use according to the fifth aspect, the subject is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the pharmaceutical composition for use according to the fifth aspect, the method is as defined in any aspect or embodiment of the present disclosure.

In a sixth aspect, the present disclosure provides a first binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject i) the first binding agent comprising at least one binding region binding to CD27; and ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In one embodiment of the first binding agent for use according to the sixth aspect, the method is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the first binding agent for use according to the sixth aspect, the first binding agent is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the first binding agent for use according to the sixth aspect, the second binding agent is as defined in any aspect or embodiment of the present disclosure.

In a seventh aspect, the present disclosure provides a second binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject i) a first binding agent comprising at least one binding region binding to CD27; and ii) the second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

In one embodiment of the second binding agent for use according to the seventh aspect, the method is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the second binding agent for use according to the seventh aspect, the first binding agent is as defined in any aspect or embodiment of the present disclosure.

In one embodiment of the second binding agent for use according to the seventh aspect, the second binding agent is as defined in any aspect or embodiment of the present disclosure.

Citation of documents and studies referenced herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the contents of these documents.

The description (including the following examples) is presented to enable a person of ordinary skill in the art to make and use the various embodiments. Descriptions of specific devices, techniques, and applications are provided only as examples. Various modifications to the examples described herein will be readily apparent to those of ordinary skill in the art, and the general principles defined herein may be applied to other examples and applications without departing from the spirit and scope of the various embodiments. Thus, the various embodiments are not intended to be limited to the examples described herein and shown, but are to be accorded the scope consistent with the claims.

*Items of the present disclosure*

1. A method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject
i) a first binding agent comprises at least one binding region binding to CD27; and
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

2. The method of item 1, wherein said first binding agent comprises a heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and a light chain variable (VL) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10 and 11, respectively.

3. The method of item 1 or 2, wherein said first binding agent comprises two binding regions capable of binding to human CD27 wherein said first binding agent comprises the heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and the light chain variable (VL) region CDR1,

CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10, and 11, respectively.

4. The method of any one of the preceding items, wherein said first binding agent comprises a VH region comprising a sequence as set forth in SEQ ID NO: 4.

5. The method of any one of the preceding items, wherein said first binding agent comprises a VL region comprising a sequence as set forth in SEQ ID NO: 8.

6. The method of any one of the preceding items, wherein said first binding agent comprises the VH and VL regions comprising the sequences as set forth in SEQ ID NO: 4 and SEQ ID NO: 8, respectively.

7. The method of any one of the preceding items, wherein said first binding agent is an antibody, preferably a human or a humanized antibody.

8. The method of any one of the preceding items, wherein the antibody is a full-length antibody further comprising a light chain constant region (CL) and a heavy chain constant region (CH).

9. The method of item 8, wherein the light chain constant region is human kappa.

10. The method of item 8, wherein the light chain constant region is human lambda.

11. The method of any one of the preceding items, wherein said first binding agent further comprises a heavy chain constant region, which is of a human IgG isotype, optionally of a modified human IgG.

12. The method of item 11, wherein the human IgG or modified human IgG is selected from IgG1, IgG2, IgG3 or IgG4, such as human IgG1.

13. The method of item 11 or 12, wherein the IgG is a modified human IgG comprising one or more amino acid substitutions.

14. The method of any one of items 11 to 13, wherein the modified human IgG is a modified human IgG1 comprising one or more amino acid substitutions, such as two or more amino acid substitutions.

15. The method of any one of items 11 to 14, wherein the modified human IgG heavy chain constant region comprises at most 10 amino acid substitutions, such as at most 9, such as at most 8, such as at most 7, such as at most 6, such as at most 5, such as at most 4, such as at most 3, such as at most 2 amino acid substitutions.

16. The method of any one of items 11 to 15, wherein said substitution in the heavy chain constant region induces increased CD27 agonism compared to an identical antibody except for comprising a wild type IgG1 antibody heavy chain constant region.

17. The method of any one of items 11 to 16, wherein the amino acid residue at the position corresponding to position E345 or E430 in a human IgG1 heavy chain according to Eu numbering is selected from the group comprising: A, C, D, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y.

18. The method of any one of items 11 to 17, wherein the amino acid residue at the position corresponding to position E345 in a human IgG1 heavy chain according to Eu numbering is R.

19. The method of any one of items 11 to 18, wherein the amino acid residue at the position corresponding to position E430 in a human IgG1 heavy chain according to Eu numbering is G.

20. The method of any one of items 11 to 19, wherein the amino acid residue at the position corresponding to position P329 in a human IgG1 heavy chain according to Eu numbering is R.

21. The method of any one of items 11 to 20, wherein the amino acid residue at the positions corresponding to position E345 and P329 in a human IgG1 heavy chain according to Eu numbering are both R.

22. The method of any one of items 11 to 21, wherein the first binding agent has a pharmacokinetic profile as the parent antibody comprising a wild type IgG1 heavy chain constant region.

23. The method of any one of the preceding items, wherein the first binding agent comprises the heavy chain constant region comprising a sequence selected from the group comprising: SEQ ID No 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 27, 28, 29, 30, 31, 32, 33, 34 and 36.

24. The method of any one of the preceding items, wherein the first binding agent comprises the heavy chain constant region comprising the sequence as set forth in SEQ ID No 15.

25. The method of any one of the preceding items, wherein said first binding agent comprises a heavy chain constant region, which is modified so that the first binding agent induces one or more Fc-mediated effector functions to a lesser extent relative to a parent antibody.

26. The method of item 25, wherein the one or more Fc-mediated effector functions is decreased by at least 20%, such as by at least 30% or by at least 40%, or by at least 50% or by at least 60% or by at least 70%, or by at least 80% or by at least 90%.

27. The method of item 25 or 26, wherein the first binding agent does not induce one or more Fc-mediated effector functions.

28. The method of any one of items 25 to 27, wherein the one or more Fc-mediated effector functions is selected from the following group: complement-dependent cytotoxicity (CDC), complement-dependent cell-mediated cytotoxicity (CDCC), complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), C1q binding and FcγR binding.

29. The method of any one of items 25 to 28, wherein the first binding agent does not induce C1q binding when measured by the method of Example 8.

30. The method of any one of the preceding items, wherein the first binding agent is a monovalent antibody.

31. The method of any one of the preceding items, wherein the first binding agent is a bivalent antibody.

32. The method of any one of the preceding items, wherein the first binding agent is a monospecific antibody.

33. The method of any one of the preceding items, wherein the first binding agent is a bispecific antibody comprising a first antigen binding region capable of binding human CD27 according to any one of the preceding items and comprising a second antigen binding region capable of binding to a different epitope on human CD27 or capable of binding a different target.

34. The method of any one of the preceding items, wherein CD27 is human CD27, in particular said human CD27 comprises the sequence as set forth in SEQ ID NO: 1 or the human CD27 variant as set forth in SEQ ID NO: 2.

35. The method of any one of the preceding items, wherein said first binding agent comprises:

a. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4;
b. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8;
c. The CH region comprising the amino acid sequence set forth in SEQ ID No: 15; and
d. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

36. The method of any one of the preceding items, wherein said first binding agent comprises a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 35 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 25.

37. The method of any one of the preceding items, wherein the first binding agent is in a composition or formulation comprising acetate, sorbitol, polysorbate 80, and has a pH from 5 to 6, preferably 5.5.

38. The method of any one of the preceding items, wherein CD40 is human CD40, in particular human CD40 comprising the sequence set forth in SEQ ID NO: 62, and/or CD137 is human CD137, in particular human CD137 comprising the sequence set forth in SEQ ID NO: 63.

39. The method of any one of the preceding items, wherein a) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 44, 45, and 46, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 47, YTS , and SEQ ID NO: 48, respectively; and b) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 51, 52, and 53, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 54, GAS, and SEQ ID NO: 55, respectively.

40. The method of any one of the preceding items, wherein

a) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 49 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 50; and

b) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 56 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 57.

41. The method of any one of the preceding items, wherein the second binding agent is a multispecific antibody, such as a bispecific antibody.

42. The method of any one of the preceding items, wherein the second binding agent is in the format of a full-length antibody or an antibody fragment.

43. The method of any one of the preceding items, wherein the second binding agent is an antibody comprising a first binding arm and a second binding arm, wherein
the first binding arm comprises

i) a polypeptide comprising said first heavy chain variable region (VH) and a first heavy chain constant region (CH), and
ii) a polypeptide comprising said first light chain variable region (VL) and a first light chain constant region (CL);
and the second binding arm comprises
iii) a polypeptide comprising said second heavy chain variable region (VH) and a second heavy chain constant region (CH), and
iv) a polypeptide comprising said second light chain variable region (VL) and a second light chain constant region (CL).

44. The method of any one of the preceding items, wherein said second binding agent comprises
i) a first heavy chain and light chain comprising said first binding region capable of binding to CD40, the first heavy chain comprising a first heavy chain constant region and the first light chain comprising a first light chain constant region; and
ii) a second heavy chain and light chain comprising said second binding region capable of binding CD137, the second heavy chain comprising a second heavy chain constant region and the second light chain comprising a second light chain constant region.

45. The method of item 43 or 44, wherein (i) the amino acid in the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering is L in said first heavy chain constant region (CH), and the amino acid in the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering is R in said second heavy chain constant region (CH), or (ii) the amino acid in the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering is R in said first heavy chain, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering is L in said second heavy chain.

46. The method of any one of item 43-45, wherein the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to EU numbering are F and E, respectively, in said first and second heavy chains.

47. The method of any one of item 43-46, wherein the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain according to EU numbering are F, E, and A, respectively, in said first and second heavy chain constant regions (HCs).

48. The method of any one of items 43-47, wherein the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to EU numbering of both the first and second heavy chain constant regions are F and E, respectively, and wherein (i) the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is L, and the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is R, or (ii) the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is R, and the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is L.

49. The method of any one of items 43-48, wherein the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain according to EU numbering of both the first and second heavy chain constant regions are F, E, and A, respectively, and wherein (i) the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is L, and the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the second heavy chain constant region is R, or (ii) the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the first heavy chain is R, and the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is L.

50. The method of any one of items 43-49, wherein the constant region of said first and/or second heavy chain, such as the second heavy chain, comprises or consists essentially of or consists of an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 58 or 60 [IgG1-Fc_FEAL];

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 6 substitutions, such as at most 5 substitutions, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

51. The method of any one of items 43-50, wherein the constant region of said first and/or second heavy chain, such as the first heavy chain, comprises or consists essentially of or consists of an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 59 or 61 [IgG1-Fc_FEAR];

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 6 substitutions, such as at most 5 substitutions, at most 4, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

52. The method of any one of any one of items 43-51, wherein said second binding agent comprises a kappa (κ) light chain constant region.

53. The method of any one of any one of items 43-52, wherein said second binding agent comprises a lambda (λ) light chain constant region.

54. The method of any one of any one of items 43-53, wherein said first light chain constant region is a kappa (κ) light chain constant region or a lambda (λ) light chain constant region.

55. The method of any one of any one of items 43-54, wherein said second light chain constant region is a lambda (λ) light chain constant region or a kappa (κ) light chain constant region.

56. The method of any one of any one of items 43-55, wherein said first light chain constant region is a kappa (κ) light chain constant region and said second light chain constant region is a lambda (λ) light chain constant region or said first light chain constant region is a lambda (λ) light chain constant region and said second light chain constant region is a kappa (κ) light chain constant region.

57. The method of any one of items 52-56, wherein the kappa (κ) light chain comprises an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 16,

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 10 substitutions, such as at most 9 substitutions, at most 8, at most 7, at most 6, at most 5, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

58. The method of any one of items 53-57, wherein the lambda (λ) light chain comprises an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 17,

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 10 substitutions, such as at most 9 substitutions, at most 8, at most 7, at most 6, at most 5, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

59. The method of any one of the preceding items, wherein the second binding agent is of an isotype selected from the group consisting of IgG1, IgG2, IgG3, and IgG4.

60. The method of any one of the preceding items, wherein the second binding agent is a full-length IgG1 antibody.

61. The method of any one of the preceding items, wherein the second binding agent is an antibody of the IgG1m(f) allotype.

62. The method of an one of the preceding items wherein the second binding agent is a bispecific antibody binding to CD40 and CD137, the bispecific antibody having i) a first heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 64 and a first light chain comprising the amino acid sequence set forth in SEQ ID NO: 65 , and ii) a second heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 66 and a second light chain comprising the amino acid sequence set forth in SEQ ID NO: 67.

63. The method of any one of the preceding items, wherein

a) the first binding agent comprises a heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and a light chain variable (VL) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10, and 11, respectively;

b) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 44, 45, and 46, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 47, YTS, and SEQ ID NO: 48, respectively; and

c) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 51, 52, and 53, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 54, GAS, and SEQ ID NO: 55, respectively.

64. The method of any one of the preceding items, wherein

a) the first binding agent comprises a VH region comprising the amino acid sequence set forth in SEQ ID No: 4, a VL region comprising the amino acid sequence set forth in SEQ ID No: 8;

b) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 49 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 50; and

c) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 56 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 57.

65. The method of any one of the preceding items, wherein

a) said first binding agent is an antibody comprising a VH region comprising the amino acid sequence set forth in SEQ ID No: 4, a VL region comprising the amino acid sequence set forth in SEQ ID No: 8, a CH region comprising the amino acid sequence set forth in SEQ ID No: 15, and a CL region comprising the amino acid sequence set forth in SEQ ID No: 17;

b) said second binding agent is an antibody comprising a first binding arm and a second binding arm, the first binding arm comprising the first binding region and a second binding arm comprising the second binding region;

c) the first binding arm of the second binding agent comprises a VH region comprising the amino acid sequence set forth in SEQ ID No: 49, a VL region comprising the amino acid sequence set forth in SEQ ID No: 50; a CH region comprising the amino acid sequence set forth in SEQ ID No: 60, and a CL region comprising the amino acid sequence set forth in SEQ ID No: 16; and

d) the second binding arm of the second binding agent comprises a VH region comprising the amino acid sequence set forth in SEQ ID No: 56, a VL region comprising the amino acid sequence set forth in SEQ ID No: 57, a CH region comprising the amino acid sequence set forth in SEQ ID No: 61, and a CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

66. The method of any one of the preceding items, wherein

a) said first binding agent comprises a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 35 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 25;

b) said second binding agent is a bispecific antibody binding to CD40 and CD137, the bispecific antibody having i) a first heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 64 and a first light chain comprising the amino acid sequence set forth in SEQ ID NO: 65, and ii) a second heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 66 and a second light chain comprising the amino acid sequence set forth in SEQ ID NO: 67.

67. The method of any one of the preceding items, wherein the subject is a human subject.

68. The method of any one of the preceding items, wherein the tumor or cancer is a solid tumor.

69. The method according to any one of the preceding items, wherein said tumor is a PD-L1 positive tumor.

70. The method of any one of the preceding items, wherein the tumor or cancer is head and neck squamous cell carcinoma (HNSCC), such as HNSCC of the oral cavity, pharynx or larynx.

71. The method of item 70, wherein the HNSCC is recurrent, unresectable or metastatic.

72. The method of any one of the items 1-69, wherein the tumor or cancer is non-small cell lung cancer (NSCLC), such as a squamous or non-squamous NSCLC.

73. The method of item 72, wherein the NSCLC is recurrent, unresectable or metastatic.

74. The method of item 72 or 73, wherein the NSCLC does not have an epidermal growth factor (EGFR)-sensitizing mutation and/or anaplastic lymphoma (ALK) translocation and/or ROS1 rearrangement.

75. The method of any one of items 72-74, wherein the NSCLC is NTRK1/2/3 (neurotrophic receptor tyrosine kinase 1/2/3) fusion positive, and/or has a mutation in KRAS (KRAS proto-oncogene, GTPase), BRAF (B-Raf proto-oncogene, serine/threonine kinase), or MET (MET proto-oncogene, receptor tyrosine kinase) gene, and/or has RET (ret proto-oncogene) gene rearrangements, and the subject has received prior treatment with a respective targeted therapy.

76. The method of any one of the preceding items, wherein the subject has received prior treatment with a PD-1 inhibitor or a PD-L1 inhibitor, such as anti-PD-1 antibody or an anti-PD-L1 antibody, preferably at least two doses of the PD-1 inhibitor or the PD-L1 inhibitor.

77. The method of any one of the preceding items, wherein the subject has received prior treatment with a platinum-based therapy or an alternative chemotherapy if platinum ineligible, eg a gemcitabine-containing regimen.

78. The method of any one of the preceding items, wherein the tumor or cancer has relapsed and/or progressed after treatment, such as systemic treatment with a checkpoint inhibitor.

79. The method of any one of the preceding items, wherein the subject has received at least one prior line of systemic therapy, such as systemic therapy comprising a PD-1 inhibitor or a PD-L1 inhibitor, such as an anti-PD-1 antibody or an anti-PD-L1 antibody.

80. The method of any one of the preceding items, wherein the cancer or tumor has relapsed and/or is refractory, or the subject has progressed after treatment with a PD-1

inhibitor or a PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody, the PD-1 inhibitor or PD-L1 inhibitor being administered as monotherapy or as part of a combination therapy.

81. The method of any one of the preceding items, wherein last prior treatment was with a PD1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody, the PD-1 inhibitor or PD-L1 inhibitor being administered as monotherapy or as part of a combination therapy.

82. The method of any one of the preceding items, wherein the time from progression on last treatment with a PD-1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody is 6 months or less.

83. The method of any one of the preceding items, wherein the time from last dosing of a PD-1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody as part of last prior treatment is 6 months or less.

84. The method of any one of the preceding items, wherein the cancer or tumor has relapsed and/or is refractory, or the subject has progressed during or after
i) platinum doublet chemotherapy following treatment with an anti-PD-1 antibody or an anti-PD-L1 antibody, or
ii) treatment with an anti-PD-1 antibody or an anti-PD-L1 antibody following platinum doublet chemotherapy.

85. A kit comprising
i) a first binding agent comprising at least one binding region binding to CD27 and
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

86. The kit according to item 85, wherein the first binding agent is as defined in any one of items 1-84 and/or the second binding agent is as defined in any one of items 1-84.

87. The kit according to item 85 or 86, wherein the first binding agent, the second binding agent, and, if present, one or more additional therapeutic agents are for systemic administration, in particular for injection or infusion, such as intravenous injection or infusion.

88. The kit according to any one of items 85-87 for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject.

89. The kit for use according to item 88, wherein the tumor or cancer is as defined in any one of items 1-84, and/or the subject is as defined in any one of items 1-84, and/or the method is as defined in any one of items 1-84.

90. A pharmaceutical composition comprising
i) a first binding agent comprising at least one binding region binding to CD27;
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137; and
iii) optionally a pharmaceutical acceptable carrier.

91. The pharmaceutical composition according to item 90, wherein the first binding agent is as defined in any one of items 1-84 and/or the second binding agent is as defined in any one of items 1-84.

92. The pharmaceutical composition according to item 90 or 91 for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject.

93. The pharmaceutical composition for use according to item 92, wherein the tumor or cancer is as defined in any one of items 1-84, and/or the subject is as defined in any one of items 1-84, and/or the method is as defined in any one of items 1-84.

94. A first binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject
i) the first binding agent comprising at least one binding region binding to CD27; and
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

95. The first binding agent for use according to item 94, wherein the method is as defined in any one of items 1-84, and/or the first binding agent is as defined in any one of items 1-84, and/or the second binding agent is as defined in any one of items 1-84.

96. A second binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject
i) a first binding agent comprising at least one binding region binding to CD27; and
ii) the second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

97. The second binding agent for use according to item 96, wherein the method is as defined in any one of items 1-84, and/or the first binding agent is as defined in any one of items 1-84, and/or the second binding agent is as defined in any one of items 1-84.

Further aspects of the present disclosure are disclosed herein.

**Example 1: Generation of DuoBody-CD40x4-1BB and anti-human CD27 antibodies and Fc variants thereof**

Generation of anti-human CD27 antibodies through immunization and hybridoma generation was performed at Aldevron GmbH (Freiburg, Germany). cDNA's encoding human CD27 (full length and ECD) were cloned into Aldevron proprietary expression plasmids. Anti-CD27 antibodies were generated by immunization of OmniRat animals (transgenic rats expressing a diversified repertoire of antibodies with fully human idiotypes; Ligand Pharmaceuticals Inc.) using intradermal application of human CD27 cDNA-coated gold-particles using a hand-held device for particle-bombardment ("gene gun"). Serum samples were collected after a series of immunizations and tested by flow cytometry on HEK cells transiently transfected with the aforementioned expression plasmid for full length human CD27 expression. Antibody-producing cells were isolated from rat spleen and fused with mouse myeloma cells (Ag8) according to standard procedures. RNA from hybridomas producing CD27-specific antibody was extracted for sequencing.

Out of a panel of 71 CD27 antibodies six antibodies were selected for further characterization based on binding to primary T cells and diversity in CD27 binding competition assays in vitro. These six antibodies are named IgG1-CD27-A, IgG1-CD27-B, IgG1-CD27-C, IgG1-CD27-D, IgG1-CD27-E and IgG1-CD27-F herein. The variable regions, in some cases with single point mutations to remove amino acid residues that were considered a liability for manufacturing (e.g. free cysteines or glycosylation sites), of heavy and light chains of interest were gene synthesized and cloned into expression vectors containing the backbone sequences for human antibody light chains and a human IgG1 heavy chain.

Fc variants of the six different antibodies were generated by introduction of one or more of the following amino acid mutations, according to Eu numbering: E345R, E430G, P329R, G237A, K326A, E333A, see Tables 1 and 3 below. After functional characterization *in vitro* as described below, CD27-specific IgG1-CD27-A (VH SEQ ID NO: 4; VL SEQ ID NO: 8) was considered to have the most optimal biological properties. Sequences of the prior art CD27-targeting antibodies used herein as benchmarks have been obtained as follows: IgG1-CD27-15 (WO2012004367; SEQ ID Nos 3 and 4), IgG1-CD27-131A (WO2018/058022; SEQ ID Nos 10 and 15), IgG1-CD27-CDX1127 (WO2016145085; SEQ ID Nos: 1 and 2), and IgG1-CD27-BMS986215 (WO2019195452A1; SEQ ID Nos 8 and 9). The VH and VL sequences of a type I anti-human CD20 antibody have been described previously in WO2019/145455A1 (SEQ ID Nos 35 and 39).

DuoBody-CD40x4-1BB is a bispecific antibody, based on the DuoBody technology platform (WO2011131746A2), which binds CD40 with one arm and 4-1BB with the other arm. DuoBody-CD40x4-1BB was generated using parental clones IgG1-CD40-001 (HC SEQ ID NO: 49; LC SEQ ID NO: 50; HCDR1 SEQ ID NO: 44, HCDR2 SEQ ID NO: 45, HCDR3 SEQ ID NO: 46, LCDR1 SEQ ID NO: 47, LCDR2: YTS, LCDR3 SEQ ID NO: 48) and IgG1-CD137-009 (HC SEQ ID NO: 56; LC SEQ ID NO: 57; HCDR1 SEQ ID NO: 51, HCDR2 SEQ ID NO: 52, HCDR3 SEQ ID NO: 53, LCDR1 SEQ ID NO: 54, LCDR2: GAS, LCDR3 SEQ ID NO: 55). As a control antibody, anti-HIV gp120 antibody IgG1-b12 was used in this application (Barbas et al., J Mol Biol 1993 230: 812-823; VH: SEQ ID NO 37, VL: SEQ ID NO 41 of this application).

*Table 1 – list of amino acid sequences*

| **SEQ ID NO:** | **Identifier** | **Amino acid sequence** |
|---|---|---|
| 1 | Human CD27 | MARPHPWWLCVLGTLVGLSATPAPKSCPERHYWAQGKLCCQMCEPG<br>TFLVKDCDQHRKAAQCDPCIPGVSFSPDHHTRPHCESCRHCNSGLLV<br>RNCTITANAECACRNGWQCRDKECTECDPLPNPSLTARSSQALSPHP<br>QPTHLPYVSEMLEARTAGHMQTLADFRQLPARTLSTHWPPQRSLCSS<br>DFIRILVIFSGMFLVFTLAGALFLHQRRKYRSNKGESPVEPAEPCHYSC<br>PREEEGSTIPIQEDYRKPEPACSP |
| 2 | CD27-A59T variant | MARPHPWWLCVLGTLVGLSATPAPKSCPERHYWAQGKLCCQMCEPG<br>TFLVKDCDQHRK**T**AQCDPCIPGVSFSPDHHTRPHCESCRHCNSGLLV<br>RNCTITANAECACRNGWQCRDKECTECDPLPNPSLTARSSQALSPHP<br>QPTHLPYVSEMLEARTAGHMQTLADFRQLPARTLSTHWPPQRSLCSS<br>DFIRILVIFSGMFLVFTLAGALFLHQRRKYRSNKGESPVEPAEPCHYSC<br>PREEEGSTIPIQEDYRKPEPACSP |
| 3 | Cyno CD27 | MARPHPWWLCFLGTLVGLSATPAPKSCPERHYWAQGKLCCQMCEPG<br>TFLVKDCDQHRKAAQCHPCIPGVSFSPDHHTRPHCESCRHCNSGLLI<br>RNCTITANAVCACRNGWQCRDKECTECDPPPNPSLTTWPSQALGPH<br>PQPTHLPYVNEMLEARTAGHMQTLADFRHLPARTLSTHWPPQRSLCS |

| | | |
|---|---|---|
| | | SDFIRILVIFSGMFLVFTLAGTLFLHQQRKYRSNKGESPMEPAEPCPYS CPREEEGSTIPIQEDYRKPEPASSP |
| 4 | CD27-A VH | QVQLMQSGSELKKPGASVKVSCRASGYTFTTYAMNWVRQAPGQGPE WMGWINTNTGNPTYAQGFTGRFVFSLDTTVTTTYLQISSLKAEDTAV YFCAREAGSFDYWGQGTLVTVSS |
| 5 | CD27-A VH CDR1 | GYTFTTYA |
| 6 | CD27-A VH CDR2 | INTNTGNP |
| 7 | CD27-A VH CDR3 | AREAGSFDY |
| 8 | CD27-A VL | QSALTQPASVSGSPGQSITISCTGTSSDVYYYNYVSWYQQHPGRAPK LVIYDVSNRPSGVSNRFSGSKSGNTASLTISGLRAEDEADYYCSSYTV NRVWVFGGGTKLTVL |
| 9 | CD27-A VL CDR1 | SSDVYYYNY |
| 10 | CD27-A VL CDR2 | DVS |
| 11 | CD27-A VL CDR3 | SSYTVNRVWV |
| 12 | Constant region human IgG1m(f) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 13 | Constant region human IgG1-E345R | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR**R**PQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 14 | Constant region human IgG1-P329R | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKAL**R**APIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 15 | Constant region human IgG1-delK E345R+P329R | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKAL**R**APIEKTISKAKGQPR**R**PQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 16 | Human kappa LC | RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC |

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| 17 | Human lambda LC | GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSP VKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGST VEKTVAPTECS |
| 18 | Constant region human IgG1m(f)-delK | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 19 | Constant region human IgG1m(a) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK PVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDE LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 20 | Constant region human IgG1m(x) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK PVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPGK |
| 21 | Constant region human IgG2 | ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVD KTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQ DWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 22 | Constant region human IgG3 | ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVD KRVELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRC PEPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTFRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKTKGQPREPQVYTLPPSREEM TKNQVSLTCLVKGFYPSDIAVEWESSGQPENNYNTTPPMLDSDGSFF LYSKLTVDKSRWQQGNIFSCSVMHEALHNRFTQKSLSLSPGK |
| 23 | Constant region human IgG4 | ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVD KRVESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK |
| 24 | CD27-A IgG1 HC with E345R+P329R | QVQLMQSGSELKKPGASVKVSCRASGYTFTTYAMNWVRQAPGQGPE WMGWINTNTGNPTYAQGFTGRFVFSLDTTVTTTYLQISSLKAEDTAV YFCAREAGSFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH |

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| | | NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL**R**API EKTISKAKGQPR**R**PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGK |
| 25 | CD27-A LC | QSALTQPASVSGSPGQSITISCTGTSSDVYYYNYVSWYQQHPGRAPK LVIYDVSNRPSGVSNRFSGSKSGNTASLTISGLRAEDEADYYCSSYTV NRVWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDF YPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTVAPTECS |
| 26 | Mouse CD27 | MAWPPPYWLCMLGTLVGLSATLAPNSCPDKHYWTGGGLCCRMCEPG TFFVKDCEQDRTAAQCDPCIPGTSFSPDYHTRPHCESCRHCNSGFLIR NCTVTANAECSCSKNWQCRDQECTECDPPLNPALTRQPSETPSPQPP PTHLPHGTEKPSWPLHRQLPNSTVYSQRSSHRPLCSSDCIRIFVTFSS MFLIFVLGAILFFHQRRNHGPNEDRQAVPEEPCPYSCPREEEGSAIPIQ EDYRKPEPAFYP |
| 27 | Human IgG1-Fc-E345R+P329R | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKAL**R**APIEKTISKAKGQPR**R**PQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 28 | Constant region human IgG1m(f) (without c-terminal lysine) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 29 | Constant region human IgG1-E345R (without c-terminal lysine) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR**R**PQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 30 | Constant region human IgG1-P329R (without c-terminal lysine) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKAL**R**APIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 31 | Constant region human IgG1m(a) (without c-terminal lysine) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK PVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDE LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |

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| 32 | Constant region human IgG1m(x) (without c-terminal lysine) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK PVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPG |
| 33 | Constant region human IgG2 (without c-terminal lysine) | ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVD KTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQ DWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 34 | Constant region human IgG3 (without c-terminal lysine) | ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVD KRVELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRC PEPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTFRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKTKGQPREPQVYTLPPSREEM TKNQVSLTCLVKGFYPSDIAVEWESSGQPENNYNTTPPMLDSDGSFF LYSKLTVDKSRWQQGNIFSCSVMHEALHNRFTQKSLSLSPG |
| 35 | CD27-A IgG1 HC with E345R+P329R (without c-terminal lysine) | QVQLMQSGSELKKPGASVKVSCRASGYTFTTYAMNWVRQAPGQGPE WMGWINTNTGNPTYAQGFTGRFVFSLDTTVTTTYLQISSLKAEDTAV YFCAREAGSFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL**R**API EKTISKAKGQPR**R**PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPG |
| 36 | Human IgG1-Fc-E345R+P329R (without c-terminal lysine) | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKAL**R**APIEKTISKAKGQPR**R**PQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 37 | VH gp120-b12 | QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWVRQAPGQRFE WMGWINPYNGNKEFSAKFQDRVTFTADTSANTAYMELRSLRSADTA VYYCARVGPYSWDDSPQDNYYMDVWGKGTTVIVSS |
| 38 | VH gp120-b12 CDR1 | GYRFSNFV |
| 39 | VH gp120-b12 CDR2 | INPYNGNK |
| 40 | VH gp120-b12 CDR3 | ARVGPYSWDDSPQDNYYMDV |
| 41 | VL gp120-b12 | EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQHKPGQAPRLV IHGVSNRASGISDRFSGSGSGTDFTLTITRVEPEDFALYYCQVYGASS YTFGQGTKLERK |

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| 42 | VL gp120-b12 CDR1 | HSIRSRR |
| | VL gp120-b12 CDR2 | GVS |
| 43 | VL gp120-b12-CDR3 | QVYGASSYT |
| 44 | CD40-001 VH CDR1 | GYTFTEYI |
| 45 | CD40-001 VH CDR2 | IIPNNGGT |
| 46 | CD40-001 VH CDR3 | TRREVYGRNYYALDY |
| 47 | CD40-001 VL CDR1 | QGINNY |
| | CD40-001 VL CDR2 | YTS |
| 48 | CD40-001 VL CDR3 | QQYSNLPYT |
| 49 | CD40-001 VH humanized | EVQLVQSGAEVKKPGASVKVSCKTSGYTFTEYIMHWVRQAPGQGLE WMGGIIPNNGGTSYNQKFQGRVTMTVDKSTSTGYMELSSLRSEDTA VYYCTRREVYGRNYYALDYWGQGTLVTVSS |
| 50 | CD40-001 VL humanized | DIQMTQSPSSLSASVGDRVTITCSASQGINNYLNWYQQKPGKAVKLL IYYTSSLHSGVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYSNLPY TFGGGTKVEIK |
| 51 | CD137-009 VH CDR1 | GFSLNDYW |
| 52 | CD137-009 VH CDR2 | IDVGGSL |
| 53 | CD137-009 VH CDR3 | ARGGLTYGFDL |
| 54 | CD137-009 VL CDR1 | EDISSY |
| | CD137-009 VL CDR2 | GAS |
| 55 | CD137-009 VL CDR3 | HYYATISGLGVA |
| 56 | CD137-009 VH humanized | EVQLVESGGGLVQPGRSLRLSCTASGFSLNDYWMSWVRQAPGKGLE WVGYIDVGGSLYYAASVKGRFTISRDDSKSIAYLQMNSLKTEDTAVY YCARGGLTYGFDLWGQGTLVTVSS |
| 57 | CD137-009 VL humanized | DIVMTQSPSSLSASVGDRVTITCQASEDISSYLAWYQQKPGKAPKRLI YGASDLASGVPSRFSASGSGTDYTFTISSLQPEDIATYYCHYYATISGL GVAFGGGTKVEIK |
| 58 | IgG1-FEAL-Fc | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPE**FE**GGPSVFLFPPKPKDTLMISRTPEVTCV VV**A**VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF **L**LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |

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| 59 | IgG1-FEAR-Fc | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPE**FE**GGPSVFLFPPKPKDTLMISRTPEVTCV VV**A**VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYS**R**LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK |
| 60 | IgG1-FEAL-Fc without C-terminal Lys | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCV VVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF LLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 61 | IgG1-FEAR-Fc without C-terminal Lys | ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCV VVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSRLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG |
| 62 | Human CD40 mature protein | EPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECLPCGESEFL DTWNRETHCHQHKYCDPNLGLRVQQKGTSETDTICTCEEGWHCTSE ACESCVLHRSCSPGFGVKQIATGVSDTICEPCPVGFFSNVSSAFEKCH PWTSCETKDLVVQQAGTNKTDVVCGPQDRLRALVVIPIIFGILFAILLV LVFIKKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAAPVQETLHGCQ PVTQEDGKESRISVQERQ |
| 63 | Human CD137 mature protein | LQDPCSNCPAGTFCDNNRNQICSPCPPNSFSSAGGQRTCDICRQCK GVFRTRKECSSTSNAECDCTPGFHCLGAGCSMCEQDCKQGQELTKK GCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLVNGTKERDVVCGPS PADLSPGASSVTPPAPAREPGHSPQIISFFLALTSTALLFLLFFLTLRFSV VKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL |
| 64 | CD40-001 heavy chain | EVQLVQSGAEVKKPGASVKVSCKTSGYTFTEYIMHWVRQAPGQGLE WMGGIIPNNGGTSYNQKFQGRVTMTVDKSTSTGYMELSSLRSEDTA VYYCTRREVYGRNYYALDYWGQGTLVTVSSASTKGPSVFPLAPSSKS TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPC PAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPG |
| 65 | CD40-001 light chain | DIQMTQSPSSLSASVGDRVTITCSASQGINNYLNWYQQKPGKAVKLL IYYTSSLHSGVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYSNLPY TFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC |
| 66 | CD137-009 heavy chain | EVQLVESGGGLVQPGRSLRLSCTASGFSLNDYWMSWVRQAPGKGLE WVGYIDVGGSLYYAASVKGRFTISRDDSKSIAYLQMNSLKTEDTAVY YCARGGLTYGFDLWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV PSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEFEG |

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| | | GPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPG |
| 67 | CD137-009 light chain | DIVMTQSPSSLSASVGDRVTITCQASEDISSYLAWYQQKPGKAPKRLI YGASDLASGVPSRFSASGSGTDYTFTISSLQPEDIATYYCHYYATISGL GVAFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK HKVYACEVTHQGLSSPVTKSFNRGEC |
| 68 | Human CD40 precursor | MVRLPLQCVLWGCLLTAVHPEPPTACREKQYLINSQCCSLCQPGQKL VSDCTEFTETECLPCGESEFLDTWNRETHCHQHKYCDPNLGLRVQQK GTSETDTICTCEEGWHCTSEACESCVLHRSCSPGFGVKQIATGVSDT ICEPCPVGFFSNVSSAFEKCHPWTSCETKDLVVQQAGTNKTDVVCGP QDRLRALVVIPIIFGILFAILLVLVFIKKVAKKPTNKAPHPKQEPQEINFP DDLPGSNTAAPVQETLHGCQPVTQEDGKESRISVQERQ |
| 69 | Human CD40 mature protein | EPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECLPCGESEFL DTWNRETHCHQHKYCDPNLGLRVQQKGTSETDTICTCEEGWHCTSE ACESCVLHRSCSPGFGVKQIATGVSDTICEPCPVGFFSNVSSAFEKCH PWTSCETKDLVVQQAGTNKTDVVCGPQDRLRALVVIPIIFGILFAILLV LVFIKKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAAPVQETLHGCQ PVTQEDGKESRISVQERQ |
| 70 | Human CD137 precursor | MGNSCYNIVATLLLVLNFERTRSLQDPCSNCPAGTFCDNNRNQICSPC PPNSFSSAGGQRTCDICRQCKGVFRTRKECSSTSNAECDCTPGFHCL GAGCSMCEQDCKQGQELTKKGCKDCCFGTFNDQKRGICRPWTNCS LDGKSVLVNGTKERDVVCGPSPADLSPGASSVTPPAPAREPGHSPQII SFFLALTSTALLFLLFFLTLRFSVVKRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFPEEEEGGCEL |
| 71 | IgG1-CD27-A-P329R-E345R-LNluc | QSALTQPASVSGSPGQSITISCTGTSSDVYYYNYVSWYQQHPGRAPK LVIYDVSNRPSGVSNRFSGSKSGNTASLTISGLRAEDEADYYCSSYTV NRVWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDF YPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTVAPTECS**SLGSSGVFTLEDFVGDWRQ TAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKID IHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVI DGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERL INPDGSLLFRVTINGVTGWRLCERILA** |
| 72 | IgG1-CD27-A-P329R-E345R-LHalo | QSALTQPASVSGSPGQSITISCTGTSSDVYYYNYVSWYQQHPGRAPK LVIYDVSNRPSGVSNRFSGSKSGNTASLTISGLRAEDEADYYCSSYTV NRVWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDF YPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTVAPTECS**SLEPTTEDLYFQSDNDGSEI GTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSY VWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFM DAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAFME FIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTL PMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEP ANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAK SLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISG** |
| 73 | IgG1-b12-P329R- | EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQHKPGQAPRLV IHGVSNRASGISDRFSGSGSGTDFTLTITRVEPEDFALYYCQVYGASS YTFGQGTKLERKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA |

| | | |
|---|---|---|
| | E345R-LNLuc | KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC**SLGSSGVFTLEDFVGDWRQTA GYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIH VIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVID GVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLI NPDGSLLFRVTINGVTGWRLCERILA** |
| 74 | IgG1-b12-P329R-E345R-LHalo | EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQHKPGQAPRLV IHGVSNRASGISDRFSGSGSGTDFTLTITRVEPEDFALYYCQVYGASS YTFGQGTKLERKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC**SLEPTTEDLYFQSDNDGSEIG TGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYV WRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMD AFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAFMEFI RPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPM GVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANI VALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLP NCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISG** |
| 75 | IgG1-CD27-A-LNLuc | QSALTQPASVSGSPGQSITISCTGTSSDVYYYNYVSWYQQHPGRAPK LVIYDVSNRPSGVSNRFSGSKSGNTASLTISGLRAEDEADYYCSSYTV NRVWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDF YPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTVAPTECS**SLGSSGVFTLEDFVGDWRQ TAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKID IHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVI DGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERL INPDGSLLFRVTINGVTGWRLCERILA** |
| 76 | IgG1-CD27-A-LHalo | QSALTQPASVSGSPGQSITISCTGTSSDVYYYNYVSWYQQHPGRAPK LVIYDVSNRPSGVSNRFSGSKSGNTASLTISGLRAEDEADYYCSSYTV NRVWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDF YPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTVAPTECS**SLEPTTEDLYFQSDNDGSEI GTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSY VWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFM DAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAFME FIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTL PMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEP ANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAK SLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISG** |
| 77 | IgG1-CD20-11B8-E430G-LNLuc | EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLI YDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSDWPL TFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC**SLGSSGVFTLEDFVGDWRQTA GYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIH VIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVID GVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLI NPDGSLLFRVTINGVTGWRLCERILA** |
| 78 | IgG1-CD37-37.3-E430G-LHalo | DIQMTQSPASLSVSVGETVTITCRASENIRSNLAWYQQKQGKSPQLL VNVATNLADGVPSRFSGSGSGTQYSLKINSLQSEDFGTYYCQHYWG TTWTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK HKVYACEVTHQGLSSPVTKSFNRGEC**SLEPTTEDLYFQSDNDGSEI** |

| | | |
|---|---|---|
| | | **GTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSY VWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFM DAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAFME FIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTL PMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEP ANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAK SLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISG** |

**Example 2: Agonist activity of anti-CD27 antibodies in a CD27 activation reporter cell assay**

CD27 agonist activity of the different anti-CD27 antibodies with and without an E345R or an E430G hexamerization-enhancing Fc mutation was measured using the CD27 Thaw and Use Bioassay kit (Promega, Custom Assay Services, CAS # CS1979A25). The kit contains NF-κB Reporter-Jurkat recombinant cells expressing the firefly luciferase gene under the control of NF-κB response elements with constitutive expression of human CD27 and was used essentially according to the manufacturer's instructions. Briefly, Thaw-and-Use GloResponse NFκB-luc2/CD27 Jurkat cells were thawed and incubated in 96-well flat bottom culture plates (PerkinElmer, Cat # 6005680) with antibody dilution series (final concentration range 0.04 - 20 µg/mL) in Bio-Glo Luciferase Assay Buffer for 6h at 37°C, 5% $CO_2$. The anti-CD27 antibodies were wild-type (WT*) IgG1-CD27-A, IgG1-CD27-B, IgG1-CD27-C, IgG1-CD27-D, IgG1-CD27-E, IgG1-CD27-F, and variants of each one harboring the E430G or E345R mutation. Anti-CD27 benchmark antibodies were IgG1-CD27-131A (WT and E430G variant) and a non-hexamerizing IgG1-CD27-15 (IgG1-CD27-15-P329R-E345R-K439E, that carries a combination of Fc mutations that prevents hexamerization and thus the mutations are functionally irrelevant in the context of this experiment and is therefore referred to as WT in the figure) and a hexamerizing variant of IgG1-CD27-15 comprising a E345R mutation. An anti-HIV gp120 human antibody, IgG1-b12-E345R, was used as a non-binding negative control antibody (ctrl). After the antibody incubation, Bio-Glo Luciferase Assay Reagent (equilibrated to RT) was added to each well and incubated at RT for 5-10 min. Luminescence was measured using an EnVision Multilabel Reader (PerkinElmer) and presented as relative luminescence units (RLU) in bar diagrams generated using GraphPad Prism software.

Introduction of a hexamerization-enhancing Fc mutation (E345R or E430G) resulted in enhanced CD27 agonism compared to the corresponding WT antibody for antibody clones IgG1-CD27-A to –E and for the benchmark antibodies IgG1-CD27-131A (tested with E430G) and IgG1-CD27-15 (tested with E345R; Figure 1).

Whereas IgG-CD27-A, B and C demonstrated enhanced CD27 agonist activity after introduction of E430G or E345R at all concentrations tested, IgG1-CD27-D and E variant

containing hexamerization-enhancing mutations did not show increased agonism at the lowest antibody concentrations. IgG1-CD27-F variants with the E430G or E345R mutations only showed enhanced CD27 agonism at the highest antibody concentration tested. For variants IgG1-CD27-A to -E, introduction of the E345R mutation resulted in stronger CD27 activation than the E430G mutation. Antibodies IgG1-CD27-A to -E having the E345R mutation showed higher or similar CD27 activation levels compared to IgG1-CD27-131A having the E430G mutation or CD27-15 having the E345R mutation, respectively.

*The WT antibodies for IgG1-CD27-B and IgG1-CD27-F carried a F405L mutation in the IgG Fc domain, which is functionally irrelevant in the context of this experiment.

**Example 3 Binding affinities of anti-human CD27 antibodies for recombinant human, mouse and cynomolgus monkey CD27**

The binding affinities of five anti-human CD27 IgG1 antibodies (IgG1-CD27-A, -B, -C, -D and –E) for recombinant human, cynomolgus monkey and mouse CD27 protein were determined using label-free biolayer interferometry on an Octet HTX instrument (FortéBio, Portsmouth, UK). Experiments were performed using bispecific antibodies comprising one CD27-specific Fab-arm and a non-binding Fab-arm, so that the antibody is monovalent for CD27. These bispecific antibodies were generated by controlled Fab-arm exchange between the CD27 antibodies and non-binding antibodies (as described in Labrijn AF et al., Nat Protoc. 2014 Oct;9(10):2450-63).

To determine the affinity of the CD27 antibodies for human and mouse CD27, 100 nM recombinant His-tagged mouse or human CD27 protein (Sino Biological, Cat # 10039-H08B1 [human], Cat # 50110-M08H [mouse]) was loaded to pre-conditioned anti-Penta-HIS (HIS1K) biosensors (FortéBio, Cat # 18-5120) for 600 sec.

To assess the affinity of the CD27 antibodies for cynomolgus monkey CD27, 5 µg/mL of recombinant cynomolgus monkey CD27-Fc fusion protein (R&D systems, Cat # 9904-CD-100) was loaded to activated Amine Reactive 2nd Generation (AR2G) biosensors (FortéBio, Cat # 18-5092).

After baseline measurements in Sample Diluent (FortéBio, Cat # 18-1104) for 300 sec, the association (200 sec) and dissociation (1,000 sec) of CD27 antibodies was determined for antibody concentration series of 0.78 – 800 nM with two-fold dilution steps in Sample Diluent. An antibody molecular mass of 150 kDa was used for calculations. Reference sensors were incubated with Sample Diluent.

Data were acquired using Data Acquisition Software v11.1.1.19 (FortéBio) and analyzed with Data Analysis Software v9.0.0.14 (FortéBio). Data traces were corrected per antibody by subtraction of the reference sensor. The Y-axis was aligned to the last 10 sec of the baseline and Interstep Correction alignment to dissociation and Savitzky-Golay filtering were applied. Data traces were excluded from analysis when the response was < 0.05 nm and calculated equilibrium was near to saturation (Req/Rmax > 95% using a dissociation time of 50 sec). The data was fitted with the 1:1 model using a window of interest for the association set at 200 sec and dissociation time set at 50 sec. The dissociation time was chosen based on the coefficient of determination ($R^2$), which is an estimate of the goodness of the curve fit (preferentially > 0.98), visual inspection of the curve, and at least 5% signal decay during the association step.

Affinities for human CD27 could be accurately determined for three CD27 antibodies (IgG1-CD27-A, -B, -C) with $K_D$ values in the nanomolar range (Table 2). For IgG1-CD27-D, and -E, BioLayer Interferometry experiments confirmed binding to human CD27 with affinities in a similar range, although suboptimal curve fitting did not allow calculation of accurate $K_D$ values (as indicated in Table 2).

IgG1-CD27-A and -B also showed binding to recombinant cynomolgus monkey CD27, with $K_D$ values in the same range as for human CD27. Results obtained with IgG1-CD27-C, -D and -E also confirmed binding to cynomolgus monkey CD27 with affinities in a similar range, although suboptimal curve fitting did not allow calculation of accurate $K_D$ values (as indicated in Table 2).

Binding to recombinant mouse CD27 was only observed for antibody IgG1-CD27-C.

Table 2. Binding affinities of IgG1-CD27-A to –E antibodies to CD27 from the indicated species.

| **Sample** | **Loading sample** | **$K_D$ (M)** | **$k_{on}$ (1/Ms)** | **$k_{dis}$ (1/s)** |
|---|---|---|---|---|
| IgG1-CD27-A | Human CD27-His | 1.3E-07 | 1.3E+05 | 1.8E-02 |
| | Mouse CD27-His | n.b. | | |
| | Cyno CD27-Fc | 1.2E-07 | 1.7E+05 | 2.0E-02 |
| IgG1-CD27-B | Human CD27-His | 5.4E-08 | 3.3E+05 | 1.8E-02 |
| | Mouse CD27-His | n.b. | | |
| | Cyno CD27-Fc | 3.5E-08 | 6.5E+05 | 2.3E-02 |
| IgG1-CD27-C | Human CD27-His | 7.0E-08 | 1.4E+05 | 9.8E-03 |
| | Mouse CD27-His | 4.3E-07 | 5.0E+04 | 2.2E-02 |
| | Cyno CD27-Fc | 5.1E-08* | 1.2E+05* | 6.4E-03* |
| IgG1-CD27-D | Human CD27-His | 4.5E-08* | 1.4+05* | 6.4E-03* |
| | Mouse CD27-His | n.b. | n.b. | n.b. |
| | Cyno CD27-Fc | 2.1E-08* | 2.4E+05* | 5.0E-03* |
| IgG1-CD27-E | Human CD27-His | 4.9E-08* | 1.3+05* | 6.4E-03* |
| | Mouse CD27-His | n.b. | n.b. | n.b. |

| | | | | |
|---|---|---|---|---|
| | Cyno CD27-Fc | 3.5E-08* | 1.6E+05* | 5.5E-03* |

* : Binding was observed but KD, $k_{on}$ and $k_{dis}$ are less reliable values due to suboptimal curve fitting, resulting in unreliable interpretation using the 1:1 model.

n.b.: no binding observed.

**Example 4: Binding of anti-CD27 antibodies to cell surface-expressed human and cynomolgus monkey CD27**

Binding of anti-CD27 antibodies IgG1-CD27-A to -E* and prior art IgG1-CD27-131A* to cell surface-expressed human and cynomolgus monkey CD27 was analyzed by flow cytometry using transiently transfected HEK293F cells and primary T cells, which endogenously express CD27. Non-binding control antibody IgG1-b12-FEAR was used as negative control antibody.

FreeStyle 293-F suspension cells (HEK293F; ThermoFisher, Cat # R79007) were transiently transfected with mammalian expression vector pSB encoding full length human or cynomolgus monkey CD27 using 293fectin Transfection Reagent (ThermoFisher, Cat # 12347019) according to the manufacturer's instructions.

Human and cynomolgus monkey PBMC were purified from buffy coats obtained from human healthy donors (Sanquin Blood Bank, the Netherlands) or from a cynomolgus monkey (BPRC, the Netherlands, Cat # S-1135) by low density gradient centrifugation using Lymphocyte Separation Medium (LSM; Corning, Cat # 25-072CV) according to the manufacturer's instructions.

Cells were seeded in 96-wells plates (100,000 cells per well; Greiner Bio-one, Cat # 650180) for sequential incubations, with washing steps in between with FACS buffer, consisting of PBS (Lonza, Cat # BE17-517Q) + 1% BSA (Roche, Cat # 10735086001) + 0.02% Sodium Azide (Bio-World, Cat # 41920044-3). The following incubations were applied: antibody concentration series (0.0001 - 10 µg/mL final concentration) for 30 min at 4°C; live/dead marker FVS510 (BD, Cat # 564406, diluted 1:1,000 in PBS) for 20 min at RT; PE-labeled polyclonal goat anti-human IgG (Jackson Immuno Research, Cat # 109-116-098, diluted 1:500) for 30 min at 4°C; and anti-CD3 antibody for T-cell identification (anti-human CD3: BD, Cat # 555335, diluted 1:10; anti-cyno CD3: Miltenyi, Cat # 130-091-998, diluted 1:10) for 30 min at 4°C. All samples were analyzed on a FACSCelesta flow cytometer (BD) and FlowJo software. Data were processed and visualized using GraphPad Prism.

All tested antibodies showed dose-dependent binding to human CD27, both on human T cells and transfected HEK293F cells (Figure 2 A,B). Highest maximal binding was observed for IgG1-CD27-B and IgG1-CD27-C compared to intermediate binding for IgG1-CD27-A and IgG1-CD27-131A, and low binding for IgG1-CD27-D and IgG1-CD27-E, with the differences

being most pronounced using human T cells. For binding to cynomolgus money CD27 T cells, highest binding was observed for IgG1-CD27-B, followed by Ig1-CD27-131A and IgG1-CD27-A. Lower binding was observed for IgG1-CD27-D and -E, whereas IgG1-CD27-C showed minimal binding to cynomolgus monkey T cells. All CD27 antibodies showed dose-dependent binding to HEK cells transfected with cynomolgus monkey CD27. Highest maximal binding was observed for IgG1-CD27-B and IgG1-CD27-131-A, somewhat lower binding was observed for IgG1-CD27-A, -D and -E. IgG1-CD27-C showed the lowest binding to HEK cells transfected with cynomolgus monkey CD27 (Figure 2 C,D).

In conclusion, IgG1-CD27-A and IgG1-CD27-B showed dose-dependent binding to human and cynomolgus monkey CD27 expressed endogenously on human or cynomolgus monkey T cells, and transiently expressed in transfected HEK cells. IgG1-CD27-A and IgG-CD27-131A showed comparable binding to human T cells, whereas IgG1-CD27-B showed higher maximal binding.

*N.B. IgG1-CD27-A, -B, -C, -D and -E carried mutations F405L-L234F-L235E-D265A in the IgG Fc domain, which are functionally irrelevant in the context of this experiment. IgG1-CD27-131A carried a functionally irrelevant F405L mutation in the IgG1 Fc domain.

**Example 5: Binding of anti-CD27 antibodies to a natural human CD27-A59T variant**

Approximately 19% of the human population expresses a natural CD27 variant harboring an A59T mutation in the extracellular domain (SEQ ID NO. 2). Binding to human CD27-A59T was tested by flow cytometry for anti-CD27 antibodies IgG1-CD27-A, IgG1-CD27-B, IgG1-CD27-C* and benchmark IgG1-CD27-131A. Non-binding antibody IgG1-b12-FEAL was used as a negative control antibody. Transiently transfected HEK293F cells expressing human CD27-A59T (15,000 cells per well) were incubated with concentration series (0.0001 – 10 µg/mL using 10-fold dilution steps) of primary test antibodies IgG1-CD27-A to -C, non-binding control antibody IgG1-b12 (ctrl), and the prior art benchmark IgG-CD27-131A, which has been described previously to bind to CD27-A59T (WO2018/058022). After incubation, antibodies were PE-labeled with polyclonal goat anti-human IgG. Binding was analyzed on a FACSCelesta flow cytometer (BD) and FlowJo software. Data were processed and visualized using GraphPad Prism v.8.

The tested anti-CD27 antibodies IgG1-CD27-A, IgG1-CD27-B, IgG1-CD27-C, and IgG1-CD27-131A showed dose-dependent binding to CD27-A59T-transfected HEK293F cells with similar binding curves among the different antibodies (Figure 3).

*N.B. IgG1-CD27-A, -B and -C carried mutations F405L-L234F-L235E-D265A in the IgG Fc domain, which are functionally irrelevant in the context of this experiment. IgG1-CD27-131A carried a functionally irrelevant F405L mutation in the IgG1 Fc domain.

**Example 6: Induction of human T cell proliferation by anti-CD27 antibodies**

As enhanced IgG hexamerization through Fc-Fc interactions upon introduction of the E345R or E430G mutation enhanced CD27 agonist activity of anti CD27 antibodies (Example 2), the capacity of IgG1-CD27-A, IgG1-CD27-B, and IgG1-CD27-C antibody variants carrying the E430G or E345R mutations to increase proliferation of TCR activated T cells was tested *in vitro*.

Additionally, Fc mutations that were reported to reduce binding to C1q and FcγR (G237A or P329R) or that enhance binding to C1q (K326A/E333A double mutation) were introduced to test their potential effect on CD27 agonist activity of CD27 antibodies carrying the E345R or E430G mutations. The K326A/E333A double mutation was previously shown to enhance C1q binding and to contribute to enhanced agonistic activity of DR5-specific humanized IgG1 antibodies comprising an Fc-Fc interaction enhancing mutation (WO2018/146317A1). The mutations G237A, P329R, or K326A/E333A were introduced, in addition to E430G or E345R, to IgG1-CD27-A, IgG1-CD27-B and IgG1-C (Table 3) and their effect on T-cell proliferation was determined using human PBMCs obtained from healthy donors (Sanquin Blood Bank, the Netherlands).

Table 3. Mutations in the Fc domain of antibodies IgG1-CD27-A, IgG1-CD27-B, or IgG1-CD27-C and their biological effect

| Fc mutation | E430G | E345R | P329R | G237A | K326/E333A |
|---|---|---|---|---|---|
| Described effect | Enhanced hexamerization | Enhanced hexamerization | Reduced C1q/FcγR binding | Reduced C1q/FcγR binding | Enhanced C1q binding |
| Antibody* | | | | | |
| IgG1-CD27-X-E430G | + | | | | |
| IgG1-CD27-X-P329R-E430G | + | | + | | |
| IgG1-CD27-X-G237A-E430G | + | | | + | |
| IgG1-CD27-X-K326A-E333A-E430G | + | | | | + |
| IgG1-CD27-X-E345R | | + | | | |
| IgG1-CD27-X-P329R-E345R | | + | + | | |
| IgG1-CD27-X-G237A-E345R | | + | | + | |

| | | | | | |
|---|---|---|---|---|---|
| IgG1-CD27-X-K326A-E333A-E345R | | + | | | + |

*X in IgG1-CD27-X, refers to IgG1-CD27 clones IgG1-CD27-A, IgG1-CD27-B, or IgG1-CD27-C.

PBMCs were resuspended in PBS at a density of 5 x $10^6$ cells/mL and labeled with CFSE using CellTrace CFSE Cell Proliferation Kit (Invitrogen, Cat # C34564; 1:10,000), according to the manufacturer's instructions. CFSE-labeled PBMCs (100,000 cells/well) were incubated in 96-well round-bottom plates (Greiner Bio-one, Cat # 650180) with 0.1 µg/mL anti-CD3 antibody clone UCHT1 (Stemcell Technologies, Cat # 60011) to activate T cells, and CD27 antibodies (1 µg/mL final concentration) in T-cell Activation Medium (ATCC, Cat # 80528190) supplemented with 5% Normal Human Serum (NHS; Sanquin, Product # B0625) for 96 h at 37°C/5% $CO_2$. For identification of viable cells in $CD4^+$ and $CD8^+$ T-cell subsets by flow cytometry, cells were sequentially incubated with live/dead marker FVS510 (1:1,000) for 20 min at RT and a staining mix for lymphocyte markers, containing APC-eFluor780-labeled anti-human CD4 antibody (Invitrogen, Cat # 47-0048-42, 1:50), AlexaFluor700-labeled anti-human CD8a antibody (BioLegend, Cat # 301028; 1:100), PE-Cy7-labeled mouse anti-human CD14 antibody (BD Biosciences, Cat # 557742; 1:50) and BV785-labeled anti-human CD19 antibody (BioLegend, Cat # 363028; 1:50) for 30 min at 4°C in the dark. Samples were measured on a FACSCelesta (BD Biosciences) flow cytometer and CFSE dilution peaks in the viable $CD4^+$ and $CD8^+$ T-cell subsets ($FVS510^-CD14^-CD19^-CD4^+$ and $FVS510^-CD14^-CD19^-CD8^+$) were analyzed using FlowJo 10 software as a readout for T-cell proliferation. T-cell proliferation was expressed as the percentage of proliferated cells or the division index both calculated by using the FlowJo software (version 10). Percentage of proliferated (divided) cells was determined by gating for the cells that have gone through CFSE dilution ($CFSE^{low\ peaks}$). The division index is the average number of divisions that the cells underwent. Heatmaps were generated using GraphPad Prism version 8. Proliferation assays were performed using PBMCs from four different healthy donors.

Variants of IgG1-CD27-A, -B and -C carrying an E430G or E345R mutation induced a small increase in proliferation of $CD8^+$ T cells compared to control antibody in two out of the four donors tested. The introduction of additional mutations (P329R, G237A or K326A/E333A) into IgG1-CD27-A, -B or -C variants carrying an E430G mutation showed variable effects on $CD8^+$ T cell proliferation across the four PBMC donors. In contrast, introduction of the P329R mutation into IgG1-CD27-A and IgG1-CD27-C variants carrying an E345R mutation consistently increased their capacity to enhance proliferation of activated $CD8^+$ T cells. This particularly applied to IgG1-CD27-A: whereas the measured $CD8^+$ T cell proliferation was

comparable for IgG-CD27-A-E345R, IgG1-CD27-B-E345R and IgG1-CD27-C-E345R in each of the donors, introduction of an additional P329R mutation consistently led to a higher increase in $CD8^+$ T cell proliferation for clone IgG1-CD27-A-E345R compared to IgG1-CD27-B-E345R or IgG1-CD27-C-E345R. Thus, the effect of the E345R mutation in combination with the P329R mutation on proliferation of TCR activated $CD8^+$ T cells was consistently larger for clone IgG1-CD27-A than for IgG1-CD27-B and IgG1-CD27-C. Across all antibody variants tested, IgG1-CD27-A-E345R-P329R induced the largest increase in $CD8^+$ T cell proliferation in all donors (Figure 4A).

The addition of the mutations G237A or K326A-E333A into CD27 antibody variants carrying the E345R mutation did not or only minimally increase the proliferation of $CD8^+$ T cells in any of the clones tested, as compared to antibodies comprising the single mutations E345R (Figure 4A).

Also in $CD4^+$ T cells, the highest and most consistent increase in T cell proliferation was observed in presence of IgG1-CD27-A-E345R-P329R (Figure 4B). Whereas $CD4^+$ T cell proliferation was generally comparable between IgG1-CD27-A, -B and -C variants carrying only the E430G or E345R mutations, introduction of an additional P329R mutation led to a larger increase in $CD4^+$ T cell proliferation for the IgG1-CD27-A variant carrying the E345R variant compared to IgG1-CD27-A-E430G or IgG1-CD27-B or -C variants carrying either the E430G or the E345R mutation. This effect was observed in three out of four donors tested. In donor 1, the effect of additional mutations in addition to E430G or E345R on $CD4^+$ T cell proliferation was generally small, and effects observed in this donor were not reproduced in the other three donors.

The combination of the E345R with the P329R mutations also consistently increased $CD4^+$ T cell proliferation for IgG1-CD27-C, although the difference between the E345R mutation alone and the combination of E345R and P329R was smaller for clone IgG1-CD27-C than for clone -A. For clone IgG1-CD27-B, a modest increase in $CD4^+$ T cell proliferation was observed for IgG1-CD27-B-E345R-P329R compared to IgG1-CD27-B-E345R in two out of the four donors.

Introduction of the P329R, G327A or K326A/E333A mutations into IgG1-CD27-A, -B, or -C variants carrying the E430G mutation did not, or not consistently, induce effects on $CD4^+$ T cell proliferation. Similarly, no or inconsistent effects were observed after introduction of the G327A or K326A/E333A in IgG1-CD27-A, -B or -C variants carrying the E345R mutation.

In summary, IgG1-CD27-A-E345R-P329R consistently induced the highest increase in proliferation of activated $CD8^+$ and $CD4^+$ T cells, demonstrating that IgG1-CD27-A-E345R-P329R induces most efficient CD27 agonism. DR5-specific, hexamerization-enhanced

antibodies with the P329R mutation previously showed reduced capacity to induce DR5 agonism compared to DR5-specific hexamerization-enhanced antibodies without the P329R mutation (Overdijk et al, Mol Canc Ther 2020). It was thus considered surprising that introduction of the P329R mutation in addition to the E345R mutation in IgG1-CD27-A enhanced CD27 agonist activity. Moreover, it is not known why the combined effect of the E345R+P329R mutations was consistently larger for IgG1-CD27-A than for IgG1-CD27-B or IgG1-CD27-C.

**Example 7: Induction of human T-cell proliferation by anti-CD27 antibody IgG1-CD27-A-P329R-E345R**

The capacity of IgG1-CD27-A-P329R-E345R to increase proliferation of TCR stimulated human $CD4^+$ and $CD8^+$ T-cells was analyzed in CSFE dilution assays using human healthy donor PBMCs, and compared to prior art anti-CD27 clones IgG1-CD27-131A*, IgG1-CD27-CDX1127, and IgG1-CD27-BMS986215*. The T-cell proliferation assays were performed as described in Example 6, with minor deviations (75,000 cells/well; concentration range 0.002 – 10 µg/mL). Samples using T cells without anti-CD3 stimulation were included to test potential CD27 agonist activity of the antibodies in absence of T-cell receptor activation (Figure 5A and 5B). Such activity is unwanted as it would pose a safety risk if the antibody was able to induce proliferation of resting T cells.

Percentage of proliferated T cells (Figure 5A, B, C, D) was calculated as the percentage of cells with reduced CFSE fluorescence, indicating cell divisions using FlowJo software. Expansion index (Figure 5E and 5F) identifies the fold increase of cells in the wells and was calculated using the Proliferation Modeling tool in FlowJo version 10. Manual adjustments to the peaks were made where necessary to define the number of the peaks present more consistently.

None of the CD27 antibodies of the invention and the prior art antibodies tested here induced proliferation of unstimulated T cells (i.e. in absence of CD3 crosslinking (Figure 5A and B).

Most of the CD27 antibodies induced some proliferation of activated $CD4^+$ and $CD8^+$ T-cells at the highest antibody concentrations tested (Figure 5C and D). Based on this, an expansion index was calculated (Figure 5E and F). Antibody IgG1-CD27-A-P329R-E345R of the invention more profoundly enhanced proliferation of $CD4^+$ and $CD8^+$ T cells *in vitro* compared to the prior art anti-CD27 clones IgG1-CD27-131A, IgG1-CD27-CDX1127 and IgG1-CD27-BMS986215.

*For IgG1-CD27-131A and IgG1-CD27-BMS986215, variants carrying a F405L mutation, that is functionally irrelevant in the context of this experiments, were used.

**Example 8: C1q binding to membrane-bound CD27 antibodies**

The P329R mutation was previously described to reduce interaction of IgG1 antibodies with C1q and FcγR (Overdijk et al, Molecular Cancer Therapeutics 2020). The effect of the P329R mutation on C1q binding of IgG1-CD27-A comprising the E345R mutation was tested in cellular C1q binding assays *in vitro* using human healthy donor T cells. Anti-HIV gp120 antibody IgG1-b12-F405L was used as non-binding isotype control antibody (ctrl). T cells were enriched from human healthy donor PBMCs using RosetteSep Human T cell Enrichment cocktail (Stemcell, Cat # 15061) and resuspended in culture medium (RPMI 1640 [Gibco, Cat # A10491-01] supplemented with 0.1% BSA and 1% Pen/Strep [Lonza, Cat # DE17-603E]). T cells (2 x $10^6$ cells/well) were pre-incubated in polystyrene 96-well round-bottom plates with antibody dilution series (8x five-fold dilution starting at 15 µg/mL final assay concentration) for 15 min at 37°C to allow the antibodies to bind to the T cells. Then, cells were cooled on ice, supplemented with NHS as a source of human C1q (20% NHS final assay concentration) and incubated on ice for 45 min. Cells were subsequently incubated on ice with FITC-labeled Rabbit anti-human C1q antibody (DAKO, Cat # F0254; 20 µg/mL) for 30 min and resuspended in FACS buffer with TO-PRO-3 (ThermoFisher, Cat # T3605; 1:5,000 dilution). C1q binding was determined by flow cytometry measuring the FITC signal on live cells.

Membrane bound WT IgG1-CD27-A antibody did not show C1q binding (Figure 6). Introduction of the hexamerization-enhancing mutation E430G or E345R (IgG1-CD27-A-E430G and IgG1-CD27-A-E345R) resulted in binding of C1q to CD27 antibodies on the T-cell surface, in line with the increased binding avidity of the hexameric C1q protein to hexameric antibody ring structures on the cell surface (Figure 6). Introduction of the P329R mutation in IgG1-CD27-A-E345R (IgG1-CD27-A-P329R-E345R) resulted in loss of C1q binding (Figure 6), demonstrating that IgG1-CD27-A-P329R-E345R was unable to bind C1q.

These data show that IgG1-CD27-A-P329R-E345R is unable to bind C1q upon binding to CD27 on the cell surface of T cells. This indicates that C1q binding does not contribute to antibody-induced CD27 agonist activity of IgG1-CD27-A-P329R-E345R. This is in contrast to what was previously described for other hexamerization-enhanced agonistic antibodies. Moreover, lack of C1q binding indicates that IgG1-CD27-A-P329R-E345R is unable to activate the classical pathway of complement activation. Thus, IgG1-CD27-A-P329R-E345R is not expected to induce complement activation and CDC on T cells which activity would be unwanted.

**Example 9: Binding of anti-CD27 antibodies to human Fc receptors**

Binding of IgG1-CD27-A- P329R-E345R to human FcγR variants was analyzed using a Biacore surface plasmon resonance (SPR) system and compared to an anti-HIV gp120 antibody IgG1-b12 (ctrl). Biacore Series S Sensor Chips CM5 (Cytiva, Cat # 29104988) were covalently coated with anti-His antibody using amine-coupling and His capture kits (Cytiva, Cat # BR100050 and Cat # 29234602) according to the manufacturer's instructions. Next, 125 nM Fcγ-receptor FcγRIa, FcγRIIa (167-His [H] and 167-Arg [R]), FcγRIIb or FcγRIIIa (176-Phe [F] and 176-Val [V]) (Sino Biological, Cat # 10256-H08S-B, Cat # 10374-H27H, Cat # 10374-H27H1-B, Cat # 10259-H27H-B, Cat # 10389-H27H-B and Cat # 10389-H27H1-B) in HBS-P+ (Cytiva, Cat # BR100827) were captured onto the surface. After three cycles of buffer, antibody samples were injected for 36 cycles to generate binding curves using antibody ranges of 0-3,000 nM for FcγRI and 0-10,000 nM for the other FcγRs. Each sample that was analyzed on an FcR-coated surface (Active Surface) was also analyzed on a parallel flow-cell without FcR (Reference Surface), which was used for background correction. Dissociation from the anti-His-coated surface was performed by regeneration of the surface using 10 mM Glycine-HCl pH 1.5 (Cytiva, Cat # BR100354). Sensograms were generated using Biacore Insight Evaluation software (Cytiva) and a four-parameter logistic (4PL) fit was applied to calculate relative binding of IgG1-CD27-A-P329R-E345R against the reference sample (ctrl).

Binding of IgG1-CD27-A-P329R-E345R to high affinity receptor FcγRIa was strongly reduced compared to the ctrl antibody, although some binding was observed at higher antibody concentrations (Figure 7A). IgG1-CD27-A-P329R-E345R did not bind to the human low affinity receptors FcγRIIa (Figure 7B and C), FcγRIIb (Figure 7D) and FcγRIIIa (Figure 7E and F).

In conclusion, IgG1-CD27A-P329R-E345R shows minimal (FcγRIa) or no (FcγRIIa, FcγRIIb, and FcγRIIIa) binding to human IgG Fc receptors.

**Example 10: Binding of anti-CD27 antibody IgG1-CD27-A-E345R-P329R to human T cells**

Binding of IgG1-CD27-A-P329R-E345R to CD27 on human healthy donor T cells was characterized in more detail using flow cytometry. Anti-HIV gp120 antibody variant IgG1-b12-P329R-E345R was used as non-binding control antibody (ctrl). Human PBMCs were isolated from buffy coats obtained from human healthy donors. PBMCs ($1 \times 10^5$ cells/well) in FACS buffer were added to polystyrene 96-well round-bottom plates (Greiner bio-one, Cat # 650101) and pelleted by centrifugation at 300xg for 3 min at 4°C. The cells were resuspended in 50 µL/well serial antibody dilutions in FACS buffer (range 0.0015 to 10 µg/mL in 3-fold dilution steps) and incubated for 30 min at 4°C. Cells were pelleted, washed twice with FACS

buffer and incubated in 50 µL/well with FITC-conjugated secondary antibody (FITC AffiniPure F(ab')$_2$ fragment goat anti-human IgG, F(ab')$_2$ fragment specific, Jackson ImmunoResearch, Cat # 109-096-097, diluted 1:100) for 30 min at 4°C in the dark. Cells were pelleted again, washed twice with FACS buffer and incubated for 30 min at 4°C in the dark in 50 µL/well of a staining mix for lymphocyte markers, containing BV711-labeled anti-human CD19 antibody (BioLegend, Cat # 302246, 1:50), AlexaFluor700-labeled anti-human CD8a antibody (BioLegend, Cat # 301028, 1:100), APC-eFluor780-labeled anti-human CD4 antibody (Invitrogen, Cat # 47-0048-42, 1:50), PE-CF594-labeled mouse anti-human CD56 antibody (BD Biosciences, Cat # 564849, 1:100), PE-Cy7-labeled mouse anti-human CD14 antibody (BD Biosciences, Cat # 557742, 1:50) and eFluor450-labeled anti-human CD3 antibody (Invitrogen, Cat # 48-0037-42, 1:200). Cells were pelleted again, washed twice using FACS buffer, and resuspended in 80 µL FACS buffer containing death cell marker 7-Amino-Actinomycin D (7-AAD; BD Biosciences, Cat # 51-68981E, 1:240 diluted). The samples were measured by flow cytometry on an LSRFortessa (BD) flow cytometer and analyzed using FlowJo software. Binding curves were analyzed using non-linear regression (sigmoidal dose-response with variable slope) using GraphPad Prism 8 software.

Anti-CD27 antibody IgG1-CD27-A-P329R-E345R showed dose-dependent binding to healthy donor T cells, with similar binding characteristics for $CD4^+$ and $CD8^+$ T cells (Figure 8).

**Example 11: FcγR-independent induction of CD27 cell signaling by anti-CD27 antibody IgG1-CD27-A-P329R-E345R**

A CD27-specific monoclonal antibody that can induce CD27 signaling independent of secondary FcγR-mediated cross-linking may be immunostimulatory in the absence of FcγR-positive cells, which would be an advantage in tumors where the frequency of FcγR-bearing cells is low.

CD27 agonist activity of IgG1-CD27-A-P329R-E345R was tested in the presence or absence of FcγR-bearing cells and compared to the corresponding WT antibody IgG1-CD27-A and prior art antibodies IgG1-CD27-131A*, IgG1-CD27-CDX1127, and IgG1-CD27-BMS986215*. Non-binding antibody IgG1-b12-P329R-E345R was used as a negative control (ctrl). CD27 reporter assays were performed, essentially as described in Example 2, with the exception that in the current example, Thaw-and-Use GloResponse NFκB-luc2/CD27 Jurkat cells were cultured in the presence of human FcγRIIb-expressing cells that can facilitate FcγR-mediated crosslinking of membrane-bound antibodies.

Thaw-and-Use effector FcγRIIb CHO-K1 cells (Promega, Cat # JA2251) were plated in 96-well flat bottom culture plates (PerkinElmer, Cat # 0815), undiluted or at three increasing dilutions

(1/3, 1/9. 1/27) and incubated overnight at 37°C / 5% $CO_2$. Supernatants of the adherent FcγRIIb-expressing cells was replaced by a Thaw-and-Use NFκB-luc2/CD27 Jurkat cell suspension of a fixed cell concentration in Bio-Glo Luciferase Assay Buffer (starting at a NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1 ratio of 1:1 for undiluted FcγRIIb CHO-K1 cells), containing serial dilutions of antibody (final concentration range 0.0002 – 10 µg/mL). After 6 h incubation at 37°C / 5% $CO_2$, plates were equilibrated to RT and bioluminescence was measured and presented as RLU as described in Example 2.

IgG1-CD27-A-P329R-E345R induced dose-dependent CD27 activation, which was independent of FcγRIIb-expressing cells (Figure 9A). In contrast, the corresponding WT antibody IgG1-CD27-A, without the E345R hexamerization-enhancing mutation and the P329R mutation, only showed CD27 agonism in the presence of FcγRIIb-expressing cells (Figure 9A-E). Similarly, CD27 activation by the prior art antibodies IgG1-CD27-131A, IgG1-CD27-CDX1127 and IgG1-CD27-BMS986215 was also dependent on the presence of FcγRIIb-expressing cells and decreased gradually with decreasing NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1 ratios (Figure 9 F-J).

In conclusion, these data indicate that IgG1-CD27-A-P329R-E345R can induce CD27 agonism independent of secondary FcγR-mediated cross-linking. This is in contrast to prior art anti-CD27 antibodies that were dependent on the presence of FcγR-bearing cells to induce CD27 agonism.

*For IgG1-CD27-131A and IgG1-CD27-BMS986215, variants carrying a F405L mutation, that is functionally irrelevant in the context of this experiment, were used.

**Example 12: Pharmacokinetic (PK) analysis of anti-CD27 antibody IgG1-CD27-A-P329R-E345R in absence of target binding, studied in mice**

The pharmacokinetic characteristics of anti-CD27 antibody IgG1-CD27-A-P329R-E345R*, in absence of target binding, was analyzed in mice and compared to the corresponding WT antibody IgG1-CD27-A*. IgG1-CD27-A does not bind to mouse CD27 (Example 3, Table 2), and thus the experiment was designed to test pharmacokinetic behaviour of IgG1-CD27-A and IgG1-CD27-A-P329R-E345R *in vivo*, in absence of target binding. The study was carried out by Crown Bioscience (China) by qualified personnel, in accordance with the approved IACUC protocol and Crown Bioscience, Inc. Standard Operating Procedures. 11-12 weeks old female SCID mice (C.B-17, Vital River Laboratory Animal Technology Co., Ltd. (VR, Beijing, China; 3 mice per group) were injected intravenously with 500 µg antibody (25 mg/kg) in a 200 µL injection volume. 40 µL blood samples were collected at 10 min, 4 h, 1 d, 2 d, 7d, 14d and 21d after antibody administration, plasma was collected from blood samples and stored

at -80°C until determination of total human IgG concentrations by ELISA. 96-well ELISA plates (Greiner, Cat # 655092) were coated overnight at 4°C with 2 µg/mL anti-human IgG (Sanquin, The Netherlands, Article # M9105, Lot# 8000260395) and subsequently blocked for 1h with PBSA (PBS supplemented with 0.2% bovine serum albumin [BSA, Roche, Cat # 10735086001]). Next, with washing steps in between, the anti-human IgG-coated plates were sequentially incubated on a plate shaker for 1h at RT with the plasma samples that were serially diluted in ELISA Buffer (PBSA supplemented with 0.05% Tween 20 [Sigma-Aldrich, Cat # P1379]), for 1h at RT with polyclonal peroxidase-conjugated goat anti-human IgG secondary antibody (Jackson, Cat # 109-035-098), and finally with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, Cat # 11112422001). The reaction was stopped by adding 2% Oxalic Acid (Riedel de Haen, Cat # 33506). Dilution series of the respective materials used for injection were used to generate reference curves. Absorbance was measured in an EL808 Microtiter plate reader (BioSPX) at 405 nm and total human IgG concentrations (in µg/mL) were plotted.

There was no substantial difference between the PK profile of IgG1-CD27-A-P329R-E345R and the counterpart WT antibody IgG1-CD27-A (Figure 10), as determined by measuring plasma IgG levels at different timepoints after intravenous injection in mice.

Although a steeper decline in the initial (distribution) phase was observed for IgG1-CD27-A-P329R-E345R and its WT counterpart (IgG1-CD27-A) compared to predictions for human IgG1 in mice, the terminal elimination of both antibodies was in line with predictions rates for human wild-type IgG1 based on a 2-compartment model (Bleeker WK, Teeling JL, Hack CE. Blood. 2001 Nov 15;98(10):3136-42).

Together, this demonstrates that introduction of the P329R and E345R mutations did not affect the pharmacokinetics properties of IgG1-CD27-A in absence of target binding.

N.B. the experiment described in this example used variants of IgG1-CD27-A and IgG1-CD27-A-P329R-E345R carrying a F405L mutation, which is functionally irrelevant in the context of this experiment.

**Example 13: Induction of antibody-dependent cellular phagocytosis by anti-CD27 antibody IgG1-CD27-A-P329R-E345R**

Antibody-dependent cellular cytotoxicity (ADCC) is mediated primarily through FcγRIIIa expressed on NK cells, whereas antibody-dependent cellular phagocytosis (ADCP) can be mediated by monocytes, macrophages, neutrophils, and dendritic cells via FcγRI, FcγRIIa, and FcγRIII (Hayes, J. M et al 2016). To understand the effect of residual binding of anti-CD27 antibody IgG1-CD27-A-P329R-E345R to FcγRIa (Example 9) on effector functions of

FcγRIa-expressing immune cells, the capacity of IgG1-CD27-A-P329R-E345R to induce ADCP was analyzed *in vitro* using CTV-labeled $CD27^+$ Burkitt's lymphoma Daudi cells as target cells, and human monocyte-derived macrophages (hMDM) as effector cells (E:T = 2:1).

hMDMs were isolated from PBMCs by positive selection using CD14 MicroBeads (Miltenyi Biotec, cat. no. 130-050-201), according to the manufacturer's instruction. PBMCs were centrifuged (1,200 RPM, 5 min, RT) and resuspended in ice-cold monocyte isolation buffer (PBS, 0.5% BSA, 2 mM EDTA) at a density of $1.25 \times 10^7$ PBMCs/mL. 20 µL CD14 MicroBeads were added per 80 µL of PBMC suspension and incubated with agitation at 4 °C for 15 min on a rollerbank. 30 mL of ice-cold monocyte isolation buffer was added, PBMC/CD14 MicroBeads mixtures centrifuged (300×g, 10 min, 4 °C) and resuspended in 6 mL ice-cold monocyte isolation buffer. LS columns (Miltenyi Biotec, cat. no. 130-042-401) were rinsed with 3 mL ice-cold monocyte isolation buffer and each column loaded with 3 mL PBMC/CD14 MicroBeads mixtures. After flow through of the $CD14^-$ cells and three washes of the column in ice-cold monocyte isolation buffer, $CD14^+$ monocytes were recovered in 3 mL of ice-cold monocyte isolation buffer by using a plunger. The $CD14^+$ cells were counted on a Cellometer Auto 2000 Cell Viability Counter (Nexcelom Bioscience) using ViaStain™ Viability Dye acridine orange/propidium iodide (AOPI; Nexcelom Bioscience, cat. no. CS2-0106), and resuspended at a density of $0.8 \times 10^6$ cells/mL in Celgene® GMP DC medium (CellGenix, cat. no. 20801-0500) supplemented with macrophage colony-stimulating factor (M-CSF; Gibco, cat. no. PH9501; 50 ng/mL final concentration) and 3 mL of monocyte suspension (i.e., $2.4 \times 10^6$ monocytes) in 100 $mm^2$ Nunc™ dishes with UpCell™ Surface, which allows cell harvesting by leaving plates at RT (Thermo Fisher Scientific, cat. no. 174902). After three days of incubation, 2 mL of fresh medium containing 5×M-CSF was added to the plates. After incubation for seven days (37 °C, 5% $CO_2$), macrophages were detached from the surface by leaving plates at RT for 1 to 1.5 h. Detached macrophages were pelleted by centrifugation, counted using AOPI, and resuspended at a density of $1 \times 10^6$ cells/mL in culture medium (RPMI 1640 with 10% DBSI).

Human Burkitt's lymphoma Daudi cells (ATCC® CCL-213™) were labeled using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific, cat. no. C34557), according to the manufacturer's instructions. Briefly, Cell Trace Violet (CTV) was added to a final concentration of 0.2 µM to $1 \times 10^6$ Daudi cells/mL in PBS and incubated in the dark at 37 °C for 20 min (15 mL incubation volume). 10 mL DBSI was added to inactivate unbound dye. Cells were pelleted by centrifugation (300×g, 5 min), washed in PBS, and counted with AOPI. CTV-labeled Daudi cells were resuspended at a density of $0.5 \times 10^6$ cells/mL in culture medium.

For the ADCP assay, hMDM (50,000 cells/well) and CTV-labeled Daudi cells (25,000 cells/well) were seeded together (E:T = 2:1) on ice in 96-well plates in a final volume of 150 µL culture medium and incubated with anti-CD27 antibody IgG1-CD27-A-P329R-E345R or anti-CD20 antibody IgG1-CD20 (0.000001 to 10 µg/mL concentration range in 10-fold dilutions), for 4 h (37 °C, 5% $CO_2$). After incubation, 100 µL Human BD Fc Block™ (BD Biosciences, cat. no. 564220; 1:100 in FACS buffer) was added and incubated at 4 °C for 10 min. Cells were pelleted by centrifugation (300×g, 5 min), resuspended in FACS buffer containing PE-Cy7 conjugated antihuman CD11b antibody (BioLegend, cat. no. 301322; 1:80) and TO-PRO-3 (Thermo Fisher Scientific, cat. no. T3605; 1:25,000) and incubated at 4 °C for 30 min. Cells were washed, resuspended in FACS buffer and collected and analyzed on a FACSymphony™ A3 Cell Analyzer (BD Biosciences). Data were analyzed using FlowJo software to measure viable target cell numbers and phagocytic hMDM and processed and visualized using GraphPad Prism software.

The percentage of viable Daudi cells for each condition was calculated according to the following formula:

% viable Daudi cells =

$$\left(\frac{\%\, TO\text{-}PRO\text{-}3^{-}CD11b^{-}CTV^{+} cells\ incubated\ with\ test\ antibody}{\%\, TO\text{-}PRO\text{-}3^{-}CD11b^{-}CTV^{+} cells\ incubated\ without\ antibody}\right) \times 100$$

The quantity of phagocytic hMDM for each condition was determined as

% TO-PRO-3$^{-}$CD11b$^{+}$CTV$^{+}$ cells.

IgG1-CD27-A-P329R-E345R did not increase the percentage of phagocytic hMDM or reduce the percentage of viable Daudi cells in the phagocytosis assay, using hMDM from four different human healthy donors. This demonstrates that residual FcγRIa binding did not result in FcγRIa-mediated effector functions for IgG1-CD27-A-P329R-E345R (data from representative human healthy donor shown in Figure 11). The positive control antibody IgG1-CD20 efficiently induced phagocytosis of Daudi cells, that express high levels of CD20, as demonstrated by an increase in the percentage of phagocytic hMDM and a decrease in the percentage of viable Daudi cells.

In conclusion, residual binding to FcγRIa was not sufficient to induce IgG1-CD27-A-P329R-E345R-dependent ADCP of CD27$^{+}$ cells.

**Example 14: Fluid-phase, target-independent, complement activation by anti-CD27 antibody IgG1-CD27-A-P329R-E345R as determined by measurement of C4d deposition**

Fc-Fc interaction-enhanced antibodies generally exist as monomeric IgG1 molecules in solution, and hexamerize on the cell surface upon target binding to form a C1q docking place in case of an active Fc region (Diebolder, C. A et al 2014; de Jong, R. N et al, 2016). The IgG Fc domain of anti-CD27 antibody IgG1-CD27-A-P329R-E345R is silenced by introduction of the P329R mutation, which results in lack of C1q binding to membrane-bound IgG1-CD27-A-P329R-E345R (Figure 6). To confirm that IgG1-CD27-A-P329R-E345R is unable to activate complement in solution in the absence of target binding, fluid phase, target-independent, complement activation was investigated by determination of C4d deposition, which is considered a measure for activation of the classical complement pathway. Fluid phase C4d fragment deposition by IgG1-CD27-A-P329R-E345R was analyzed by an enzyme-linked immunosorbent assay (ELISA) using the MicroVue™ C4d Enzyme Immunoassay (EIA; Quidel, cat. no. A008) and was performed according to the manufacturer's protocol. Heat Aggregated Gamma Globulin (HAGG; Complement Activator; Quidel, cat. no. A114) was used as a positive control for the assay. IgG1-b12 and IgG1-b12-RGY (WO2014006217A1)) were included as control antibodies. Introduction of E345R/E430G/S440Y (RGY) Fc mutations in an IgG1 antibody has been described to induce the formation of hexamers in solution, resulting in fluid phase complement activation (Diebolder, C. A et al, 2014; Wang, G., R. N et al, 2016; de Jong, R. N et al , 2016). IgG1-b12-P329R-E345R was included as isotype control antibody.

Antibody dilutions were prepared in phosphate-buffered saline (PBS) to a concentration of 1 mg/mL, except for HAGG, which was diluted to a concentration of 10 mg/mL. Then, the test samples were further diluted to a concentration of 100 µg/mL (for monoclonal IgG) or 1,000 µg/mL (for HAGG) in 90% (final concentration) normal human serum (NHS) (CompTech, Lot. no. 42a) and incubated at 37 °C for 1 h. In parallel, 'No antibody' samples (no antibody, 90% NHS) and 'PBS only' samples (no antibody, no NHS) were included as negative controls. Next, the samples were diluted 1:250 in cold kit-provided Complement Specimen Diluent. In the meantime, the strips coated with mouse anti-human C4d antibody were placed in a 96-wells plate and the assay wells were washed three times with 250 to 300 µL Wash Buffer with a 1-min waiting step after the first wash. The test samples were added to the wells (100 µL/well) and as a negative control, Complement Specimen Diluent only (blank) was used in the ELISA. In parallel, 100 µL of the standards (Standard A-E) and internal controls provided by the kit were added to separate wells. The plates were incubated for 30 min at RT. Then, the plates were washed five times with Wash Buffer as described above. 50 µL of C4d Conjugate (peroxidase-conjugated goat anti-human C4d) was added to the wells and the plates were incubated for 30 min at RT. After five washing

steps with Wash Buffer as described above, 100 µL of C4d Substrate [0.7% 2-2'-Azino-di-(3-ethylbenzthiazoline sulfonic acid diammonium salt] was added and again the plates were incubated for 30 min at RT. Finally, 50 µL kit-provided Stop Solution was added and within 1 h, the optical density was measured at 405 nm using an ELISA Plate Reader (EL808 BioSPX, BioTek).

IgG1-CD27-A-P329R-E345R and the control antibody IgG1-b12-P329R-E345R (having the same Fc backbone as IgG1-CD27-A-P329R-E345R) did not induce fluid phase C4d deposition at the tested concentration of 100 µg/mL; the measured C4d levels were similar to background levels of the control antibody with a wild-type Fc domain (IgG1-b12) and the no antibody control (Figure 12). In contrast, the positive control antibody IgG1-b12-RGY, that is known to form hexamers in solution, induced C4d deposition to the same level as HAGG.

These data show that IgG1-CD27-A-P329R-E345R did not induce target-independent, fluid phase complement activation *in vitro*.

**Example 15: Capacity of anti-CD27 antibody IgG1-CD27-A-P329R-E345R to compete for ligand-binding with CD70**

To determine if anti-CD27 antibody IgG1-CD27-A-P329R-E345R interferes with the interaction of CD27 with its natural ligand CD70, binding of a saturating concentration of biotinylated recombinant human CD70 extracellular domain (ECD) to CD27, endogenously expressed on human Burkitt's lymphoma cell line Daudi, was studied in the presence and absence of excess IgG1-CD27-A-P329R-E345R.

Daudi cells (ATCC® CCL-213™) cultured in RPMI 1640 medium (Gibco, cat. no. A10491-01) supplemented with 10% donor bovine serum with iron (DBSI; Gibco, cat. no. 20731-030) were seeded at 50,000 cells/well in round bottom 96-well plates (Greiner Bio One, cat. no. 650261). Cells were pelleted by centrifugation (300×g, 3 min at 4 °C) and resuspended in FACS buffer (PBS, 1% BSA [Roche, cat. no. 1073508600]) containing anti-CD27 or control antibodies (50 µg/mL final concentration). Biotinylated recombinant human CD70 ECD (Abcam, cat. no. ab271443) was added at a saturating concentration (6 µg/mL) and cells were incubated at 4 °C for 30 min.

Cells were washed twice and resuspendend in FACS buffer containing Brilliant Violet (BV) 421™ labeled streptavidin (BioLegend, cat. no. 405225; 0.0025 µg/mL final concentration)

and R phycoerythrin (PE) labeled polyclonal AffiniPure F(ab')$_2$ fragment goat-anti-human IgG Fc (Jackson ImmunoResearch, cat. no. 109 116098; 0.0025 µg/mL final concentration) at 4 °C for 30 min. Cells were washed twice, resuspended in FACS buffer containing TO-PRO-3 iodide (Thermo Fisher Scientific, cat. no. T3605; 1:25,000) and analyzed. Data were collected on a BD FACSymphony™ A3 flow cytometer (BD Biosciences) and analyzed using FlowJo software. For compensation, one drop of UltraComp eBeads™ Compensation Beads (Life Technologies, cat. no. 01-2222-42) was added to each well. 2 µL of each antibody was added and mix was incubated for 20 min. Plates were spun down and beads were resuspended in FACS buffer and measured. For viability compensation, cells were treated at 65 °C for 10 min and mixed 1:1 with viable cells. Cells were spun down and resuspended in TO-PRO-3 diluted in FACS buffer. Data were processed and visualized using GraphPad Prism.

IgG1-CD27-A-P329R-E345R or IgG1-CD27-A did not block binding of the CD70 ECD to CD27$^+$ Daudi cells, as CD70 binding levels were comparable to those for Daudi cells incubated with the nonbinding isotype control antibodies IgG1-b12-P329R-E345R or IgG1-b12, or cells without antibody (Figure 13). Also, prior art anti-CD27 antibodies IgG1-CD27-BMS986215 and IgG1-CD27-131A showed a weak blocking effect on CD27 binding to CD70 ECD. In contrast, CD70 was unable to bind to surface CD27 on Daudi cells in presence of prior art anti-CD27 antibody IgG1-CD27-CDX1127 (Figure 13) that was previously reported to block ligand-binding (Vitale et al, 2012).

In conclusion, IgG1-CD27-A-P329R-E345R binding does not block CD27 binding by its natural ligand CD70 on Daudi cells.

**Example 16: T-cell activation marker expression upon incubation of polyclonally stimulated human PBMCs with anti-CD27 antibodies**

The effect of IgG1-CD27-A-P329R-E345R on expression of T-cell activation markers in polyclonally activated T cells was studied using PBMCs obtained from three different healthy human donors. Expression of HLA-DR, CD25, CD107a, and 4-1BB were analyzed after incubating PBMCs with IgG1-CD27-A-P329R-E345R or prior art anti-CD27 antibodies for two and five days.

Freshly isolated 75,000 PBMCs/well were seeded in 96-well U bottom plates (Greiner Bio-One) in cell culture medium. Duplicate wells were incubated simultaneously with anti-CD3 antibody (UCHT1 clone; Stemcell; 0.1 µg/mL); and IgG1-CD27-A-P329R-E345R (0.0005 to

30 µg/mL in threefold dilutions); or prior art anti-CD27 antibodies IgG1-CD27-CDX1127, IgG1-CD27-131A, and IgG1-CD27-BMS986215 (30 µg/mL); or nonbinding control antibody IgG1-b12-P329R-E345R (10 µg/mL). To determine expression of each activation marker in absence of treatment, duplicate control wells with untreated (no anti-CD3 or anti-CD27 antibodies) cells were supplemented with culture medium alone. To set the gates for identifying activation marker positive cells, fluorescence minus one (FMO) controls were used. For the FMO controls, all the antibodies used in the experiment except for one corresponding to an activation marker in duplicate wells was added to 75,000 PBMCss/well from one donor activated with anti-CD3 antibody. Untreated cells from each donor in single wells with no staining antibody were included as negative controls. To detect viable cells, untreated cells from each donor were stained with 4',6-diamidino-2-phenylindole (DAPI) alone in single wells.

After incubation for two or five days (37 °C, 5% $CO_2$), plates were washed once with FACS buffer and resuspended in an antibody mixture in FACS buffer containing antibodies for T-cell activation markers 4-1BB, CD25, CD107a, human leukocyte antigen (HLA)-DR; and antibodies for gating $CD4^+$ and $CD8^+$ T-cell subsets in flow cytometry. After incubation at 4 °C for 30 min, all plates were washed twice with FACS buffer and cells were resuspended in FACS buffer. The samples were analyzed on a BD LSRFortessa Cell Analyzer using FlowJo software to determine the median fluorescence intensity (MFI) and percentage of positive cells for each T-cell activation marker on $CD4^+$ and $CD8^+$ T cells. Anti-CD27 antibody induced changes in the expression levels of the T-cell activation markers were presented as the fold change in MFI of the anti-CD27 antibody sample relative to the nonbinding control antibody IgG1-b12-P329R-E345R. The samples were analyzed on a BD LSRFortessa™ Cell Analyzer (BD Biosciences) using FlowJo software.

IgG1-CD27-A-P329R-E345R increased expression of CD25, CD107a and 4-1BB on activated $CD4^+$ T cells (Figure 14A). These effects were more pronounced after 2 days of incubation than after 5 days of incubation. On $CD8^+$ T cells, incubation with IgG1-CD27-A-P329R-E345R resulted in an increased expression of HLA-DR, CD107a and 4-1BB both after 2 and 5 days of incubation (Figure 14B).

The expression of T-cell activation markers was also assessed upon incubation for 2 and 5 days with three prior art antibodies. IgG1-CD27-131A and IgG1-CD27-BMS986215 induced a comparable increase in expression of HLA-DR, 4-1BB, CD25, and CD107a on $CD4^+$ and $CD8^+$ T cells, while the effect of incubation for 2 or 5 days with IgG1-CD27-CDX1127 on T-cell activation marker expression was less pronounced.

In conclusion, incubation of polyclonally activated PBMCs with IgG1-CD27-A-P329R-E345R resulted in an increased expression of activation markers HLA-DR, CD25, CD107a and 4-1BB on $CD4^+$ and $CD8^+$ T cells.

**Example 17: Percentages of OVA-specific $CD8^+$ T cells in OVA protein-immunized mice after injection of anti-CD27 antibodies in a human CD27-KI mouse model**

The effect of IgG1-CD27-A-P329R-E345R treatment on expansion of antigen-specific T cells in the hCD27 KI OVA model in splenocytes was analyzed by flow cytometry.

Homozygous human CD27 (hCD27)-KI mice on a C57BL/6 background (hCD27 KI mice) were obtained from Beijing Biocytogen Co., Ltd. (strain name C57BL/6-Cd27tm1(CD27)/Bcgen, Stock no. 110006). This strain was developed in collaboration with the HuGEMM™ platform of Crown Bioscience, featuring a humanized drug target (CD27 in this case) within mice with a functional immune system. In hCD27 KI mice, exons 1-5 of the mouse CD27 gene encoding the extracellular domain were replaced by human CD27 exons 1-5. OVA-specific T cells were induced *in vivo* by subcutaneous (s.c.) injection of the immunogen ovalbumin (OVA) in hCD27-KI mice and the agonist effect of IgG1-CD27-A-P329R-E345R was tested by simultaneously treating the mice intravenously (i.v.) with the antibody.

On day 0, the mice were injected s.c. with 5 mg OVA (InvivoGen, cat. no. vac-pova-100, lot. no. EFP-42-04) and treated by i.v. injection into the tail vain with IgG1-CD27-A-P329R-E345R (30 mg/kg), IgG1-CD27-CDX1127 (30 mg/kg) or IgG1-b12-P329R-E345R (30 mg/kg). On day 12 and day 21, mice were boosted with OVA and treated with antibody as on day 0. On day 10, day 19 and day 24, blood was collected via cheek pouch or saphena in BD Microtainer® blood collection tubes containing di-potassium ethylenediaminetetraacetic acid (K2-EDTA; BD, cat. no. 365974) and immediately used in further analysis. On day 28, mice were euthanized and spleens were resected under sterile conditions.

Resected spleen tissue in RPMI1640 medium (Thermo Fisher Scientific, cat. no. C22400500BT) was transferred to gentleMACs™ C Tubes (Miltenyi Biotec, cat. no. 130-093-237) and mechanically dissociated to a single cell suspension using the gentleMACS™ Dissociator (Miltenyi, cat. no. 130-093-235), according to the manufacturer's instructions. After dissociation, the cell suspension was filtered through a 70 µm cell strainer (Falcon, cat. no. 352350). Next, samples were washed twice by resuspension in 3 mL wash buffer (sterile PBS [Hyclone, SH0256.01B] supplemented with 4% FBS [Gibco, cat. no. 10099 141]). Cells

were counted on a Cellometer Auto T4 (Nexcelom Bioscience) and the number of cells was adjusted to $2 \times 10^6$ splenocytes per tube.

$2 \times 10^6$ splenocytes were transferred to FACS tubes (Falcon, cat. no. 352052) and resuspended in wash buffer (sterile PBS [Hyclone, SH0256.01B] supplemented with 4% FBS [Gibco, cat. no. 10099 141]) supplemented with 1 µg/mL purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™, BD Biosciences, cat. no. 553141). After a preincubation at 2-8 °C for 10 min in the dark, 10 µL PE-labeled OVA-tetramer (MBL Life science, cat. no. TS 5001 1C) was added, and the samples were gently vortexed before further incubating at 2-8 °C for 30-60 min in the dark. Without washing, labeled antibodies and compounds used for flow cytometry gating of T-cell subsets were added. The samples were gently vortexed and incubated at 2-8 °C for an additional 30 min in the dark. Next, samples were washed twice by resuspension in 2 mL wash buffer and centrifuged at 300×g for 5 min. Finally, the cells were resuspended in 250 µL wash buffer and analyzed on a BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences). Data were processed using Kaluza Analysis Software (Beckman Coulter).

IgG1-CD27-A-P329R-E345R increased the percentages of OVA-specific $CD8^+$ T cells in the spleen of mice simultaneously injected with OVA protein vaccination. The percentages of OVA-specific $CD8^+$ T cells in mice treated with 30 mg/kg IgG1-CD27-CDX1127 were lower than the IgG1-CD27-A-P329R-E345R-treated group and comparable to the IgG1-b12-P329R-E345R-treated group (Figure 15). Similar observations were made in peripheral blood samples.

**Example 18: IFNγ secretion by OVA-specific $CD8^+$ T cells from spleens of OVA-immunized mice injected with anti-CD27 antibodies**

Resected spleen tissue in RPMI1640 medium (see Example 17) was gently mashed over a 70 µm cell strainer (Falcon, cat. no. 352350), pelleted by centrifugation (1,500 rpm, 5 min), and resuspended in 10 mL Ammonium-Chloride-Potassium (ACK) Lysing Buffer (Invitrogen, cat. no. A1049201). After 3-5 min incubation at RT, samples were washed twice with 10-20 mL PBS and resuspended in 5 mL Cellular Technology Limited (CTL) Test™ Medium (ImmunoSpot, cat. no. CTLT-005) supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (pen/strep, Gibco, cat. no. 15070-063). The collected splenocytes were filtered again through a 70 µm cell strainer and counted on a Vi-CELL™ XR Cell Viability

Analyzer (Beckman Coulter) to adjust the concentration to 3.125 × $10^6$ cells/mL with CTL-Test Medium containing pen/strep.

IFNγ production by splenocytes was analyzed using the Mouse IFN-γ ELISpotPLUS kit (Mabtech, cat. no. 3321-4HPW-2), essentially as described by the manufacturer. Pre-coated MultiScreenHTS IP Filter (MSIP) white plates (mAb AN18) were washed four times with 200 µL sterile PBS per well and conditioned with 200 µL CTL-Test Medium containing pen/strep (RT, 30 min). Medium was removed and 5 × $10^5$ splenocytes/well were incubated in duplicate with 2 µg/mL $OVA_{257-264}$ peptide SIINFEKL (Invivogen, cat. no. vac-sin), or scrambled control peptide FILKSINE (SB-PEPTIDE, cat. no. SB073-1MG) in a total volume of 180 µL/well for 20 h in a humidified incubator (37 °C, 5% $CO_2$). As a positive control for IFNγ production, splenocytes were incubated in parallel with a cell stimulation cocktail consisting of 500 ng/mL phorbol myristate acetate (PMA) and 10 µg/mL ionomycin (PMA+Ionomycin, Dakewe Biotech, cat. no. DKW ST PI). Cultures of splenocytes without peptide were included as a negative control. After incubation, the cells were removed and the plates were washed five times with PBS. Next, plates were sequentially incubated, with five wash steps with PBS in between, with Biotinylated detection mAb (R4-6A2; RT, 2 h), Streptavidin-horseradish peroxidase (HRP; RT, 1 h), and finally 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (all provided by the kit). When distinct spots emerged, the reaction was stopped by washing extensively in deionized water. Spots were counted on an AID iSpot ELISpot Reader (Autoimmun Diagnostika [AID] GMBH, ELR08IFL) using spotAID V8 software (AID). ELISpot data were analyzed and presented in bar diagrams using GraphPad Prism software and presented as the mean number of spots per well ± SEM from all mice per treatment group (n=5).

Splenocytes from all IgG1-CD27-A-P329R-E345R-treated animal groups showed increased IFNγ production in response to treatment with OVA peptide, as demonstrated by ELISpot analysis (Figure 16). Stimulation of the splenocytes with a scrambled control peptide induced no or minimal IFNγ production, suggesting that IFNγ was produced by OVA-specific T cells. In contrast, no IFNγ production was observed in splenocytes from mice treated with 30 mg/kg IgG1-CD27-CDX1127.

**Example 19: Effect of IgG1-CD27-A-P329R-E345R treatment on T-cell activation in OVA-immunized mice *in vivo***

The effect of IgG1-CD27-A-P329R-E345R treatment on $CD8^+$ T-cell activation was studied *in vivo* by analyzing the expression of PD-1 on $CD8^+$ T cells derived from OVA-treated hCD27-KI mice. Mice were treated as described in Example 17. Also, methods to obtain and analyze splenocytes by FACS are described in Example 17.

IgG1-CD27-A-P329R-E345R induced an increase in the percentage of $CD8^+$ T cells expressing activation marker PD-1 on day 28. $CD8^+PD\text{-}1^+$ T-cell percentages were low in animals treated with IgG1-CD27-CDX1127 or control antibody IgG1-b12-P329R-E345R (Figure 17).

**Example 20: Effect of IgG1-CD27-A-P329R-E345R treatment on *in vivo* induction of T-cell subsets in OVA-immunized mice**

The effect of IgG1-CD27-A-P329R-E345R on the expansion of T-cell subsets was studied by analyzing the expression of CD44 and CD62L in splenocyte samples from OVA-treated hCD27-KI mice. Memory $CD8^+$ T cells derived from spleens of IgG1-CD27-A-P329R-E345R-treated, OVA-immunized, hCD27-KI mice were quantified by flow cytometry. Memory T cells were classified as effector memory ($CD44^+CD62L^-$) and pre-effector T cells ($CD44^-CD62L^-$; Nakajima, Y., K et al 2018). Mice were treated as described in Example 17. Also, methods to obtain and analyze splenocytes by FACS are described in Example 17.

IgG1-CD27-A-P329R-E345R (30 mg/kg) induced increased percentages of pre-effector T cells and effector memory $CD8^+$ T cells in the spleen on day 28 when compared to splenocytes of mice treated with IgG1-b12-P329R-E345R (Figure 18). Within the $CD45^+$ population, IgG1-CD27-A-P329R-E345R induced higher percentages of pre-effector T cells and effector memory T cells than IgG1-CD27-CDX1127 (30 mg/kg), while comparable mean percentages of these T-cell populations were induced by both anti-CD27 antibodies in the $CD8^+$ fraction of splenocytes.

**Example 21: Effect of IgG1-CD27-A-P329R-E345R treatment on *in vivo* expansion of T cells in OVA-immunized mice**

The effect of IgG1-CD27-A-P329R-E345R on expansion of T cells was studied by analyzing the expression of CD3 in splenocyte and blood samples from OVA-treated hCD27-KI mice. Mice were treated as described in Example 17. Also, methods to obtain and analyze splenocytes and blood samples by flow cytometry are described in Example 17.

Treatment of OVA-immunized hCD27-KI mice with 30 mg/kg IgG1-CD27-A-P329R-E345R did not increase the percentage of $CD3^+$ T cells in the spleen, compared to treatment with the non-binding control antibody IgG1-b12-P329R-E345R (Figure 19). In contrast, treatment with benchmark antibody IgG1-CD27-CDX1127 (30 mg/kg) resulted in a decrease of $CD3^+$ T cells in the spleen. Similar observations were made in peripheral blood samples.

**Example 22: Effect of IgG1-CD27-A-P329R-E345R on T-cell cytokine production in antigen-specific studies**

The capacity of IgG1-CD27-A-P329R-E345R to increase cytokine production was studied using T cells that had been stimulated by their cognate antigen. PBMC were isolated from buffy coats obtained from healthy human donors by Ficoll-Paque density gradient separation (GE Healthcare, cat. no. 17 1440 03) according to the manufacturer's instructions.

Human magnetic CD14 and CD8 MicroBeads (Miltenyi Biotec, cat. no. 130 050 201 and 130 045 201, respectively) were used for positive selection of $CD14^+$ monocytes and negative selection of $CD14^-$ PBL from human PBMC, and positive selection of $CD8^+$ T cells from frozen PBL. Cell suspensions were centrifuged and resuspended in magnetic-activated cell sorting (MACS) buffer (Dulbecco's phosphate-buffered saline [DPBS] with 5mM EDTA, 1% human albumin) at $1 \times 10^7$ live cells per 80 µL MACS buffer. Per $1 \times 10^7$ cells, 12 µL CD14 or CD8 MicroBeads were added. Subsequent MACS separation was performed using an automated magnetic cell separation instrument or by manual separation. Automated MACS separation was performed using an autoMACS® Pro Separator (Miltenyi Biotec), according to the manufacturer's instructions. Eluted $CD14^+$ monocytes and $CD8^+$ T cells were centrifuged (8 min, 300×g at RT) resuspended in X-VIVO 15 medium (Lonza), and counted with erythrosine B solution for further use; i.e., monocyte differentiation into iDC or electroporation of $CD8^+$ T cells with PD-1 and/or CLDN6-specific T-cell receptor (TCR) mRNA.

For the generation of monocyte-derived iDC, up to $40 \times 10^6$ PBMC-derived $CD14^+$ monocytes were cultured (37 °C, 5% $CO_2$) for five days in T175 flasks in DC medium (RPMI 1640, 5% pooled human serum [PHS; One Lambda, cat. no. A25761], 1× minimum essential medium non-essential amino acid solution [MEM NEAA, Life Technologies, cat. no. 11140 035], 1 mM sodium pyruvate [Life Technologies, cat. no. 11360 039]) supplemented with 100 ng/mL human granulocyte/macrophage colony-stimulating factor (GM-CSF;

Miltenyi Biotec, cat. no. 130-093-868) and 50 ng/mL human IL-4 (Miltenyi Biotec, cat. no. 130093 924). After three days in culture, half of the medium per flask was replaced. Since the medium taken from the flask contained non-adherent monocytes, it was centrifuged (8 min, 300 ×g, RT), the supernatant discarded, the cell pellet resuspended in fresh DC medium and then returned into the originator flask together with 200 ng/mL GM-CSF and 200 ng/mL IL-4 (final concentration). After the five days of incubation, the iDC which adhered to the culture flask were detached using 10 mL DPBS containing 2 mM EDTA (37 °C, 10 min). The isolated iDC were washed, pelleted (8 min, 300×g at RT) and used for electroporation with CLDN6 mRNA.

Human $CD8^+$ T cells were electroporated with RNA encoding the alpha and beta chains of a mouse TCR specific for human CLDN6, either alone or together with RNA encoding PD-1, and human monocyte-derived iDC were electroporated with RNA encoding human CLDN6. Up to $5 \times 10^6$ iDC or $15 \times 10^6$ $CD8^+$ T cells were electroporated in 250 µL X-VIVO 15 medium at RT using an ECM 830 Square Wave Electroporation System (BTX®). Cells were mixed with RNA, pulsed (500 V, 3 ms for T cells or 300 V, 12 ms for iDC), and immediately diluted with 750 µL pre-warmed assay medium (IMDM GlutaMAX [Life technologies, cat. no. 31980030] with 5% PHS). Electroporated iDC were transferred to 6- or 12-well plates and cultured O/N (37 °C, 5% $CO_2$). After O/N incubation, electroporated $CD8^+$ T cells and iDC were evaluated by flow cytometry to evaluate cell purity, expression of transfected RNA (PD-1 and CLDN6-TCR on $CD8^+$ T cells and CLDN6 on iDC), and baseline expression of CD27 and PD-1 on $CD8^+$ T cells and PD-L1 on iDC. Approximately 78% to 93%, 78% to 92%, and 36% to 98% of electroporated $CD8^+$ T cells expressed CLDN6-TCR, PD-1, and endogenous CD27, respectively. Approximately 47% to 91% and 94% to 99% of electroporated iDC expressed CLDN6 and endogenous PD-L1, respectively (not shown).

$CD8^+$ T cells and iDC were seeded at a 10:1 ratio ($7.5\times10^4$ T cells and $7.5\times10^3$ iDC per well) in a 96-well round-bottom plate. IgG1-CD27-A-P329R-E345R was diluted in assay medium and 25 µL of diluted IgG1-CD27-A-P329R-E345R was added to the wells, to reach a final concentration of 10 µg/mL. Similarly, the control antibodies IgG1-CD27-131A and IgG1-b12-P329R-E345R were added to reach final concentrations of 10 µg/mL. Antigen-specific T-cell activity upon antibody treatment was analyzed *in vitro* by measuring cytokines in the supernatant of T cells transduced to express CLDN6-TCR, which were co-cultured with iDC transduced to express and present CLDN6. Supernatants were collected after two days, and concentrations of multiple proinflammatory cytokines and chemokines were determined by multiplex electrochemiluminescence assays (ECLIA) using the 10-spot

U-PLEX ImmunoOncology Group 1 (human) kit (MSD; cat. no. K151AEL 2) following the manufacturer's instructions.

For the 10-spot U-PLEX Immuno-Oncology Group 1 kit, biotinylated capture antibodies were pre-incubated at RT with the assigned linkers, which have a biotin-binding domain, for 30 min, followed by 30 min incubation with Stop Solution. Plates were coated with a mix of the linker coupled capture antibodies by incubating at RT with shaking for 1 hr. Plates were washed three times with 1× MSD Wash Buffer. Supernatant samples or kit standards were diluted 1:2 in Assay Diluent, added to the wells and incubated at RT for 2 h with constant shaking. The plates were washed three times with Wash Buffer, and incubated with SULFO-TAG-conjugated detection antibodies from the kit at RT for 1 h with constant shaking. The plates were washed three times with Wash Buffer before adding Read Buffer B to catalyze the electrochemiluminescent reaction. The plates were immediately analyzed by measuring light intensity on a MESO QuickPlex SQ 120 imager (MSD).

IgG1-CD27-A-P329R-E345R-induced changes in cytokine production were assessed by multiplex ECLIA in supernatants from the $CD8^+$ T cell/iDC co-cultures after two days of incubation (n=4 different donors). IgG1-CD27-A-P329R-E345R induced a significant increase in the production of GM-CSF and IFNγ in $CD8^+$ T cell/iDC co-cultures with $CD8^+$ T cells expressing endogenous levels of PD-1 (Figure 20A), while also an increase in IL-13 and TNFα production was observed. A considerable increase for the same cytokines was observed in cultures containing PD-1-overexpressing T cells (Figure 20B). While cytokine levels were generally decreased when T cells overexpressed PD-1, the relative increase (fold increase) in cytokine production in presence of IgG1-CD27-A-P329R-E345R was generally higher this setting (Figure 20A and B). In contrast, prior art anti-CD27 antibody IgG1-CD27-131A showed minimal effect on cytokine production compared to the nonbinding control antibody IgG1-b12-P329R-E345R (Figure 20A and B).

**Example 23: Expression of cytotoxicity-associated molecules by antigen-specific $CD8^+$ T cells incubated with IgG1-CD27-A-P329R-E345R**

The induction of T-cell mediated cytotoxicity upon antibody treatment was studied by analyzing the expression of cytotoxicity-associated molecules on the antigen-specific T cells by flow cytometry in co-cultures of human healthy donor T cells transduced to express a CLDN6-TCR and MDA-MB-231_hCLDN6 target cells.

MDA-MB-231_hCLDN6 cells were generated by lentiviral transduction. To this end, $2\times10^5$ MDA-MB-231 cells in 250 µL Dulbecco's modified eagle medium (DMEM, Thermo Fisher

Scientific, cat. no. 31966-047) supplemented with 10% FBS (non-heat-inactivated) were seeded per well in a 12-well tissue culture plate. The cells were incubated for 1-2 h at 37 °C (7.5% $CO_2$). Supernatants containing lentiviral vectors encoding human CLDN6 (pL64b42E(EF1a-hClaudin6)Hygro-T2A-GFP) were thawed on ice and diluted in a total volume of 750 µL DMEM/10% FBS to obtain titers of $2\times10^5$, $8\times10^4$, and $3.2\times10^4$ TU/mL. These titers corresponded to MOI of 1, 0.4, and 0.16, respectively. The supernatants were then added to the MDA-MB-231 cells, and the cells were incubated for 72 h at 37 °C (5% $CO_2$) without disturbance. For the experiments described in the current Example, MDA-MB-231-hCLDN6 cells were cultured in DMEM/10% FBS. Cells were passaged or harvested for experiments at 70% to 90% confluence. Cells were detached by treatment with Accutase (Thermo Fisher Scientific, cat. no. A11105010) for 5 min (37 °C, 7.5% $CO_2$), and resuspended by addition of culture medium. Cells were centrifuged (300×g, 4 min at RT) and counted. MDA-MB-231_hCLDN6 cells were not cultured for more than 20 passages.

MDA-MB-231_hCLDN6 cells were seeded at 1.2 to 1.5 × $10^4$ cells/well, in 96-well flat-bottom plates (for flow cytometry analysis) and xCELLigence E-plates (Agilent, cat. no. 05232368001; for impedance measurement) and allowed to settle at RT for 30 min. Next, plates were incubated for one day in the incubator and the xCELLigence real-time cell analysis (RTCA) instrument (ACEA Biosciences), respectively (37 °C, 5% $CO_2$).

Isolated $CD8^+$ T cells (see Example 22) were electroporated with CLDN6-specific TCR mRNA and incubated O/N. After $CD8^+$ T-cell isolation and electroporation, T-cell cultures contained 49% to 99% $CD8^+$ T cells. Of these electroporated $CD8^+$ T cells, approximately 78% to 93% expressed CLDN6-TCR and 59% to 98% of CLDN6-$TCR^+$ $CD8^+$ cells were $CD27^+$. Cells were centrifuged (8 min, 300×g at RT), resuspended in DMEM/10% FBS and counted. The cells were centrifuged again, resuspended at 3 × $10^6$ cells/mL in DMEM/10% FBS, and added to the wells containing the previously seeded MDA-MB-231_hCLDN6 cells (1.5 × $10^5$ $CD8^+$ T cells/well; T cell:tumor cell, effector:target, ratio of 10:1). IgG1-CD27-A-P329R-E345R, IgG1-CD27-131A, and the nonbinding control antibody IgG1-b12-P329R-E345R were added to the co-cultures at 10 µg/mL. CD107a and GzmB expression were determined by flow cytometry.

After two days of incubation in the presence of 10 µg/mL IgG1-CD27-A-P329R-E345R, the percentage of $GzmB^+CD107a^+CD8^+$ T cells was significantly enhanced compared to treatment with the nonbinding control antibody or prior art anti-CD27 antibody IgG1-CD27-131A (Figure 21).

In conclusion, these data show that IgG1-CD27-A-P329R-E345R was able to induce cytotoxicity-associated molecules on activated antigen-specific T cells.

**Example 24: Capacity of IgG1-CD27-A-P329R-E345R to induce T-cell mediated tumor cytotoxicity**

To evaluate T-cell mediated cytotoxicity, CLDN6-TCR-electroporated $CD8^+$ T cells were co-cultured with MDA-MB-231_hCLDN6 cells in the presence of IgG1-CD27-A-P329R-E345R, prior art anti-CD27 antibody IgG1-CD27-131A, or nonbinding control antibody IgG1-b12-P329R-E345R for five days in an xCELLigence real-time cell analysis instrument (Acea Biosciences), with impedance measurements at two-hour intervals, as described in Example 23. Cell index values were derived from impedance measurements conducted at two-hour intervals. Area-under-the-curve (AUC) were obtained from cell index data over five days of co-culture. AUC were normalized to co-cultures treated with IgG1-b12-P329R-E345R. The magnitude of impedance is dependent on cell number, cell morphology, and cell size and on the strength of cell attachment to the plate, which altogether is used in this particular case as an indirect readout of tumor cell mass. Decrease in impedance in this experimental setting is considered a surrogate of tumor-cell killing by $CD8^+$ T cells. It should be noted that impedance may underestimate tumor cell killing due to proliferation of T cells.

IgG1-CD27-A-P329R-E345R induced a decrease in cell index, indicative of tumor-cell killing. IgG1-CD27-131A did not have a visible effect on cell index, indicating minimal capacity to increase tumor-cell killing (Figure 22).

**Example 25: Capacity of IgG1-CD27-A-P329R-E345R to induce expansion of tumor-infiltrating lymphocytes**

The capacity of IgG1-CD27-A-P329R-E345R to induce expansion of tumor-infiltrating lymphocyte (TIL) subsets ($CD4^+$ and $CD8^+$ T cells, NK cells, and regulatory T cells [Treg]) was evaluated *ex vivo* using cryopreserved tumors that had been surgically resected from NSCLC patients.

Surgically resected human NSCLC tissues were received in transport medium (HypoThermosol® FRS Preservation Solution [BioLife Solutions, cat. no. 101104], 7.5 µg/mL Amphotericin B [Thermo Fisher Scientific, cat. no. 15290026], and 300 units/mL (U/mL) pen/strep [Thermo Fisher Scientific, cat. no. 15140-122]). Samples were washed

three times in wash medium (5 mL X-VIVO 15 [Lonza], 2.5 µg/mL Amphotericin B, [Thermo Fisher Scientific] and 100 U/mL pen/strep [Thermo Fisher Scientific]) and transferred to a cell culture dish. Fatty tissue and necrotic areas were removed with a scalpel, and the tissue was cut into fragments of approximately 5 $mm^3$. Each fragment was placed in an individual cryovial, and 1 mL freezing medium (FBS, 10% DMSO) was added to each vial. The vials were transferred into a controlled freeze-chamber (Mr. Frosty freezing container), which was placed in a −80 °C freezer. After at least 16 h at −80 °C, the vials were transferred to liquid nitrogen for long-term storage.

4 to 6 cryopreserved vials containing tumor fragments of approximately 5 $mm^3$ from one tumor specimen were thawed per experiment in a 37 °C water bath for approximately 2 min and washed five times with wash medium and transferred to a cell culture dish. The tumor fragments were further dissected with a scalpel into fragments of approximately 1 $mm^3$. Most of the fragments were used for TIL expansion upon culturing with IL-2 and treatment antibody and remaining fragments were used to determine expression of specific cell surface markers at baseline, without any treatment.

Two tumor fragments per well (on average) were seeded in 24-well plates (2 mL/well total volume capacity used in assay) in 0.1 mL prewarmed TIL cultivation medium (X-VIVO 15 [Lonza] with 2% human serum albumin [HSA; CSL Behring, cat. no. PZN-00504775], 100 U/mL pen/strep [Thermo Fisher Scientific], and 2.5 µg/mL Amphotericin B [Thermo Fisher Scientific]) containing 45 to 50 U/mL IL-2 (Proleukin S; Novartis Pharma, cat. no. PZN-02238131). IgG1-CD27-A-P329R-E345R was diluted in TIL cultivation medium containing 45 to 50 U/mL IL-2 and 900 µL of this dilution was added to the wells as appropriate. Final IgG1-CD27-A-P329R-E345R concentrations in the wells were 1 or 10 µg/mL. As a control, medium containing 45 to 50 U/mL IL-2 without antibodies was added to tumor fragments in separate wells. A total of 8 to 16 wells were incubated for each experimental condition per donor (37 °C, 5% $CO_2$).

After three days of culture, fresh TIL cultivation medium containing 45 to 50 U/mL IL-2 and IgG1-CD27-A-P329R-E345R was added to the wells (1 mL/well, same antibody concentrations as above). Between day 5 and 14/17 after assay initiation, the cultures were regularly monitored with a microscope for proliferation of TIL that migrated from the tissue fragments and the formation of TIL microclusters. If >25 TIL microclusters were observed in one well after seven or eight culture days, cells and tissue fragments from two identically treated original wells were resuspended and pooled into one well of a 6-well plate (5 to 6

mL/well total volume capacity used in assay) with the culture medium and fresh IL 2 containing TIL cultivation medium was added (estimated 33 U/mL IL-2 final concentration).

Every two to three days, cultures were supplemented with fresh IL-2-containing TIL cultivation medium. IL-2 concentrations in the medium added to cultures were reduced to 10 U/mL, or first reduced to 25 U/mL and then to 10 U/mL thereafter after supplementing the wells with medium throughout the assay. On day 14 or 17, the cells were harvested for flow cytometry analysis.

IgG1-CD27-A-P329R-E345R enhanced expansion of TIL subtypes compared to control cultures treated with IL-2 alone, with the largest relative increase in cell count observed for $CD8^+$ T cells and Tregs, followed by $CD4^+$ T cells, and NK cells. For all TIL subsets, expansion was more pronounced with IgG1-CD27-A-P329R-E345R at 1 µg/mL than 10 µg/mL (Table 4 and Figure 23).

Table 4. Fold-expansion of IgG1-CD27-A-P329R-E345R-treated TIL
Tumor tissues derived from human NSCLC specimens were cultured with low-dose IL-2 in the presence or absence of IgG1-CD27-A-P329R-E345R. Absolute cell counts of the indicated cell subsets were determined by flow cytometry after 14 to 17 days of treatment. Fold differences in cell numbers for IgG1-CD27-A-P329R-E345R-treated cultures relative to cultures treated with IL-2 are shown. Data shown are from five tumor tissues from individual patients tested in five independent experiments. P=0.0236, 1 µg/mL vs. 10 µg/mL IgG1-CD27-A-P329R-E345R (two-way ANOVA).

| **Cell population** | **All TIL** | | **$CD4^+$ T cells** | | **$CD8^+$ T cells** | | **Treg** | | **NK cells** | |
|---|---|---|---|---|---|---|---|---|---|---|
| IgG1-CD27-A-P329R-E345R concentration (µg/mL) | **1** | **10** | **1** | **10** | **1** | **10** | **1** | **10** | **1** | **10** |
| Patient #578 | 14.9 | 2.1 | 19.3 | 2.3 | 19.6 | 2.9 | 86.9 | 2.1 | 11.3 | 1.1 |
| Patient #507 | 27.9 | 5.1 | 33.7 | 4.4 | 107.1 | 17.4 | 32.2 | 11.9 | 14.4 | 6.0 |
| Patient #594 | 0.6 | 1.5 | 0.4 | 1.0 | 1.8 | 2.2 | 0.4 | 2.3 | 0.8 | 2.6 |
| Patient #592 | 0.9 | 0.8 | 0.4 | 0.2 | 2.9 | 1.7 | 4.8 | 2.6 | 2.3 | 1.2 |
| Patient #561 | 0.8 | 1.6 | 0.2 | 2.9 | 0.8 | 1.0 | n.d. | n.d. | 1.1 | 1.2 |
| Average±SD [a] | 11.1 ± 11.3 | 2.4± 1.6 | 13.5 ± 14.0 | 2.0± 1.6 | 32.9 ± 43.4 | 6.1± 6.6 | 31.1 ± 34.5 | 4.9± 4.1 | 7.2± 5.8 | 2.7± 2.0 |

[a]Average and SD calculations exclude patient #561 for better comparability between cell populations.

Abbreviations: ANOVA = analysis of variance; n.d. = not determined; NK = natural killer; NSCLC = non-small cell lung cancer; SD = standard deviation; TIL = tumor-infiltrating lymphocyte; Treg = regulatory T cell.

**Example 26: BRET analysis to assess intermolecular interactions of IgG1-CD27-A-P329R-E345R molecules on the cell surface**

The capacity of CD27 antibodies harboring the hexamerization-enhancing mutation (E345R) to increase intermolecular Fc-Fc interactions after binding to CD27 on the cell surface was determined using bioluminescence resonance energy transfer (BRET) analysis. This molecular proximity-based assay detects protein interactions by measuring energy transfer from a bioluminescent protein donor to a fluorescent protein acceptor. Energy transfer occurs only when the donor and acceptor are in close proximity (<10 nm [Wu and Brand, 1994; Dacres et al, 2012]).

First, cell surface expression of CD27, as well as CD20 and CD37 (as positive control molecules), was determined on huCD27-K562, a human chronic myelogenous leukemia cell line genetically modified to stably express human CD27, and on Daudi cells, using an indirect immunofluorescence assay (QIFIKIT, Agilent Technologies, cat no. K0078). Cells were seeded at 100,000 cells/well and incubated with 10 µg/mL primary antibody (CD27: IgG1-7730-143-C102S-FEAL; CD20: IgG1-11B8-FEAR; CD37: IgG1-3009-010-FEAR). This was followed by incubation with a FITC-labeled polyclonal goat anti-human IgG (Jackson Immuno Research, cat. no. 109-096-097), in parallel with QIFIKIT beads coated with a defined number of antibody molecules. The number of antibody molecules per cell was determined by interpolating the measured mean fluorescence intensity (MFI) of a test sample on the calibration curve generated by plotting the MFI of the individual bead populations against the known number of antibody molecules per bead. Samples were measured on an LSRFortessa Cell Analyzer flow cytometer (BD Biosciences) and analyzed using FlowJo software.

QiFi analysis showed moderate CD27 expression and high CD20 and CD37 expression on Daudi cells, whereas huCD27-K562 cells expressed high levels of CD27, but no CD20 and CD37 (Table 5).

Table 5: Cell surface expression in antibody molecules per cell

| | **huCD27-K562** | **Daudi** |
|---|---|---|

| | | |
|---|---|---|
| **CD27** | 390,373 | 15,484 |
| **CD20** | - | 180,217 |
| **CD37** | - | 219,663 |

BRET assay (NanoBRET™ System, Promega, cat no. N1661) was performed essentially according to the manufacturer's instructions. To generate NanoLuc (donor) and HaloTag (acceptor) tagged antibodies, variable light chain sequences with either NanoLuc or HaloTag (Table 1, sequences 71-78) were prepared by gene synthesis, cloned into appropriate expression vectors and full-length antibodies produced as described in Example 1. For analysis, $0.5x10^5$ huCD27-K562 or Daudi cells were seeded in 96-well round-bottom plates (Greiner Bio-One, cat. no. 650101) in a total volume of 100 µL. Cells were pelleted by centrifugation (3 min at 300xg) and resuspended in 50 µL assay medium (Opti-MEM I [Gibco, cat. no. 11058-021] + 4% FBS [ATCC, cat. no. 30-2020]) containing mixtures of NanoLuc- or HaloTag-tagged antibody pairs each at a concentration of 5 µg/mL. Next, 50 µL HaloTag NanoBret 618 ligand (Promega, cat. no. G980A, 1:1000 dilution in assay medium) was added. For each antibody mixture, a no-ligand control sample was prepared in parallel, by adding 50 µL medium without HaloTag NanoBret 618 ligand. Cells were incubated for 30 min at 37°C in the dark, washed twice with medium and resuspended in 100 µL assay medium without FBS. 25 µL NanoBRET NanoGLO substrate (Promega, cat. no. N1571, 1:200 dilution in assay medium without FBS) was added to each well. Plates were shaken for 30 s and 120 µL of each sample was transferred to an OptiPlate (Perkin Elmer, cat. no. 6005299). An EnVision Multilabel Reader (Perkin Elmer) was used to measure donor emission at 460 nm and acceptor emission at 618 nm.

BRET was calculated in milliBRET units (mBU) = (618 $nm_{em}$/460 $nm_{em}$) x 1000.

Results are reported as Corrected BRET, which is corrected for donor-contributed background or bleedthrough, and calculated as: mBU ligand – mBU no-ligand control.

The proximity of NanoLuc- and HaloTag-labeled IgG1-CD27-A-P329R-E345R antibodies after binding CD27 on the cell surface was compared to WT IgG1-CD27-A antibodies carrying the same tags. IgG1-CD20-11B8-E430G-LNLuc and IgG1-CD37-37.3-E430G-LHalo antibodies, containing an E430G mutation that induces hexamerization (WO2019243636A1), were used as a positive control for proximity-induced BRET. IgG1-CD20-11B8-E430G and IgG1-CD37-37.3-E430G were previously shown to form heterohexamers upon binding to cells expressing CD20 and CD37, using molecular proximity assays (Oostindie, S.C. et al, Haematologica, 2019). Nonbinding antibody IgG1-b12-P329R-E345R was used as a negative control.

As positive and negative controls for BRET signal induction, Daudi cells (high CD20 and CD37 expression) and huCD27-K562 cells (no CD20 and CD37 expression) were opsonized with antibody pair IgG1-CD20-11B8-E430G-LNLuc and IgG1-CD37-37.3-E430G-LHalo. BRET induction was detected only on Daudi cells, and not on huCD27-K562 cells lacking CD20 and CD37 (Figure 24). Similarly, a non-binding control antibody pair (IgG1-b12-P329R-E345R-LNLuc + IgG1-b12-P329R-E345R-LHalo) did not induce BRET on either cell line. When huCD27-K562 cells were opsonized with a mixture of NanoLuc- and HaloTag-labeled CD27 antibodies bearing the hexamerization-enhancing mutation (IgG1-CD27-A-P329R-E345R-LNLuc + IgG1-CD27-A-P329R-E345R-LHalo), high BRET was detected, while BRET on Daudi cells did not exceed background levels (Figure 24). A mixture of IgG1-CD27-A-LNLuc and IgG1-CD27-A-LHalo (WT) antibodies induced considerably lower BRET on huCD27-K562 cells compared to CD27 antibodies carrying the P329R and E345R mutations, and no BRET on Daudi cells. These results indicate that BRET signal was associated with higher target expression. CD27 expression on huCD27-K562 cells was found to be ~26 fold higher than on Daudi cells, while BRET levels for CD27-binding IgG1-CD27-A-P329R-E345R on huCD27-K562 cells were ~24 fold higher than on Daudi cells. Mixtures of NanoLuc- and HaloTag-labeled nonbinding and CD27-binding antibody pairs (IgG1-b12-P329R-E345R-LNLuc + IgG1-CD27-A-P329R-E345R-LHalo, and IgG1-CD27-A-P329R-E345R-LNLuc + IgG1-b12-P329R-E345R-LHalo respectively), did not induce BRET on either cell line. This confirms that observed BRET was dependent on simultaneous interaction of donor and acceptor antibodies bound to the cell-surface target.

In summary, IgG1-CD27-A-P329R-E345R induced high BRET on huCD27-K562 cells compared to its WT variant. This finding confirms enhanced proximity between membrane-bound IgG1-CD27-A-P329R-E345R molecules, compared to its WT variant, consistent with E345R-enhanced Fc-Fc interactions between cell surface-bound antibodies.

N.B. the experiment described in this example used a variant of IgG1-CD27-A carrying a F405L mutation, which is functionally irrelevant in the context of this experiment.

## Example 27: Binding of IgG1-CD27-A-P329R-E345R to FcγRIa$^+$ M0 and M1 macrophages

Example 9 assessed binding of IgG1-CD27-A-P329R-E345R to human FcγR variants using surface plasmon resonance (SPR), showing minimal (FcγRIa) or no (FcγRIIa, FcγRIIb, and FcγRIIIa) binding to recombinant human IgG Fc receptor molecules. This residual FcγRIa binding was not sufficient to induce IgG1-CD27-A-P329R-E345R-dependent ADCP of CD27$^+$

cells (see Example 13). To further exclude interactions of IgG1-CD27-A-P329R-E345R with FcγRIa-positive macrophages, Fc-mediated binding of IgG1-CD27-A-P329R-E345R to M0 and M1 macrophages was determined.

Human $CD14^+$ monocytes were isolated from PBMCs from two healthy donors as described in Example 13, and differentiated into monocyte-derived macrophages by culturing the cells in medium (CellGenix, cat. no. 20801-0500) supplemented with 50 ng/mL M-CSF (Gibco, cat. no. PHC9501) to obtain M0 macrophages, or 50 ng/mL GM-CSF (Immunotools, cat. no. 11343125) for differentiation into M1 macrophages. After 6 days of culture, M0 and M1 phenotypes were confirmed by FACS analysis according to expression of markers as defined in Table 6. Additionally, both macrophage subtypes were confirmed to express human Fc receptors FcγRIa, FcγRII and FcγRIIIa (Table 6).

Table 6:

| **Phenotype markers** | **M0** | **M1** |
|---|---|---|
| CD40 (BD Pharmingen, cat. no. 561211, 1:50 dilution) | + | + |
| CD86 (MACS, cat. no. 30-097-877, 1:50 dilution) | + | ++ |
| CD163 (Biolegend, cat.no. 333612, 1:200 dilution) | +/- | - |
| CD206 (Biolegend, cat. no. 321136, 1:200 dilution) | +/- | + |
| **Fc receptors** | | |
| FcγRIa (Biolegend, cat. no. 305006, 1:25 dilution) | ++ | ++ |
| FcγRII (BD Pharmingen, cat. no. 552883, 1:50 dilution) | ++ | ++ |
| FcγRIIIa (BD Pharmingen, cat. no. 555407, 1:50 dilution) | + | +/- |

Binding of IgG1-CD27-A-P329R-E345R to M0 and M1 macrophages was compared to binding of a WT IgG1 antibody (IgG1-b12) with an irrelevant antigen-binding region as a positive control for FcγRIa binding, and a variant of the same antibody also carrying the P329R mutation previously described to reduce interaction with FcγR (IgG1-b12-P329R-E345R). Since macrophages should not express CD27, any binding observed is hypothesized to occur via FcγRIa, which is the only FcγR that binds monovalent IgG. The differentiated macrophages were incubated with IgG1-CD27-A-P329R-E345R or control antibodies (30 µg/mL in DC medium) for 15 min, and PE-labeled polyclonal goat anti-human IgG (Jackson Immuno Research, cat. no. 109-116-097, dilution 1:200, 30 min at 4°C). After incubation, cells were washed and resuspended in 100 µL FACS buffer containing nucleus-staining DAPI (BD

Pharmingen, cat. no. 564907, 1:5000 dilution). Samples were measured on a FACSymphony flow cytometer (BD Biosciences) and analyzed using FlowJo software.

No binding above background (secondary antibody only) to M0 or M1 macrophages isolated from two independent donors was observed with either IgG1-CD27-A-P329R-E345R or control IgG1-b12-P329R-E345R (Figure 25). WT IgG1-b12, which contains an active Fc region, consistently bound to both M0 and M1 macrophages.

In conclusion, the IgG1-CD27-A-P329R-E345R and control IgG1-b12-P329R-E345R do not bind M0 or M1 macrophages expressing FcγRIa, FcγRII and FcγRIIIa.

**Example 28: Induction of proliferation of polyclonally activated human CD8$^+$ T cells by IgG1-CD27-A-P329R-E345R in combination with DuoBody-CD40x4-1BB**

The effect of IgG1-CD27-A-P329R-E345R in combination with DuoBody-CD40x4-1BB on activated human CD8$^+$ T cells was analyzed by flow cytometry using freshly isolated human healthy donor PBMCs, in which T cells were polyclonally stimulated with a CD3 antibody.
Human peripheral blood mononuclear cells (PBMCs) were freshly isolated from human healthy donor buffy coats by low density gradient centrifugation using lymphocyte separation medium (Corning, cat. no. 25-072-CI) according to the manufacturers' instructions. PBMCs were washed twice in PBS (HyClone, cat. no. SH3A3830.03) supplemented with 2% donor bovine serum with iron (DBSI; Gibco, cat. no. 20371-030) at a density of 10 × 10$^6$ cells/mL and labeled with CTV using CellTrace™ Violet Cell Proliferation Kit (Invitrogen, cat. no. C34557A, diluted in PBS), according to the manufacturer's instructions. CTV-labeled PMBC (7.5 × 104 cells/well) were plated in round-bottom 96-well plates (Greiner Bio-One, cat. no. 650180) and mixed with anti-CD3 antibody (aCD3, clone UCHT1, final concentration in the assay 0.1 µg/mL, Stemcell, cat. no. 60011) in assay medium (RPMI 1640 [Lonza, cat. no. 12-115F], 10% donor bovine serum with iron [DBSI; Gibco, cat. no. 20731-030], 1% Pen/Strep [Lonza, cat no. DE17-603E]) to trigger T-cell activation. Subsequently, aCD3 stimulated PBMCs were incubated with IgG1-CD27-A-P329R-E345R (0.0016 – 10 µg/mL in 5-fold dilutions) and DuoBody-CD40x4-1BB (0 – 0.000064 µg/mL in 5-fold dilutions), either alone or in combination, in a total volume of 150 µL at 37°C for four days. The cell suspensions were pelleted and incubated with FACS buffer (PBS [Lonza, cat. no. BE17517Q], 0.02% sodium azide [bioWorld, cat. no. 41920044 3], 0.1% BSA [Roche, cat. no. 43279213], 2 mM EDTA [Sigma, cat. no. BCCD3789]) containing the lymphocyte marker AF700-labeled anti-human CD8 (BD BioLegend, cat. no. 301028, 1:50) at 4 °C for 30 min. Cells were washed three times with FACS buffer and resuspended in FACS buffer containing viability dye TO-PRO-3 (Invitrogen, cat. no. T3605). Flow cytometry data were acquired on a FACS Symphony (BD).

CTV dilution peaks in the viable $CD8^+$ T-cell subsets ($CD8^+$7-AAD) were analyzed using the proliferation modeling tool in FlowJo software (v10.7.3) and expansion indices were determined according to the following formula:

Number of cells at start of culture = (G0) + (G1)/2 + (G2)/4 + (G3)/8 + (G4)/16 +…(GN/2N)

Expansion index = Total cell number (sum G0 to GN) / Number of cells at start

G0 to GN are single proliferation peaks, with G0 representing the undivided cell fraction and GN the cell fraction that divided N times.

A dose-dependent increase in $CD8^+$ (Figure 26) T-cell proliferation was observed in the PBMC samples treated with IgG1-CD27-A-P329R-E345R alone over the whole antibody concentration range. The dose-response curves of the samples treated with DuoBody-CD40x4-1BB alone showed a bell-shaped curve, reaching a maximal expansion index at an antibody concentration of 1 µg/mL. The combination of IgG1-CD27-A-P329R-E345R with DuoBody-CD40x4-1BB increased $CD8^+$ T-cell proliferation more potently than each antibody alone, and the maximal effects were reached at the highest tested IgG1-CD27-A-P329R-E345R concentrations (2 to 10 µg/mL) in combination with the intermediate to high concentrations that were tested for DuoBody-CD40x4-1BB (0.04 to 5 µg/mL).

These data indicate that the combination of IgG1-CD27-A-P329R-E345R with DuoBody-CD40x4-1BB showed a higher increase in T-cell proliferation compared to each antibody alone.

**Example 29: Antigen-specific stimulation assay to determine the capacity of IgG1-CD27-A-P329R-E345R in combination with DuoBody-CD40x4-1BB to enhance T-cell proliferation**

To determine the combinatorial effect of IgG1-CD27-A-P329R-E345R and DuoBody-CD40x4-1BB on T-cell proliferation and cytokine production compared to single-agent activity, an antigen-specific proliferation assay was conducted using co-cultures of healthy human CD8+ T cells and cognate antigen-expressing immature dendritic cells (iDCs).

HLA-A*$02^+$ peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (Transfusionszentrale, University Hospital, Mainz, Germany). Monocytes were isolated from PBMCs by magnetic-activated cell sorting (MACS) technology using anti-CD14 MicroBeads (Miltenyi; cat. no. 130-050-201), according to the manufacturer's instructions. The peripheral blood lymphocytes (PBLs, CD14-negative fraction) were cryopreserved in RPMI 1640 containing 10% DMSO (AppliChem GmbH, cat. no. A3672,0050) and 10% human albumin (CSL Behring, PZN 00504775) for T-cell isolation. For differentiation into iDCs,

40 × $10^6$ monocytes/mL were cultured for five days in RPMI 1640 (Life Technologies GmbH, cat. no. 61870-010) containing 5% pooled human serum (One Lambda Inc., cat. no. A25761), 1 mM sodium pyruvate (Life technologies GmbH, cat. no. 11360-039), 1x non-essential amino acids (Life Technologies GmbH, cat. no. 11140-035), 200 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Miltenyi, cat. no. 130-093-868) and 200 ng/mL human interleukin-4 (IL-4; Miltenyi, cat. no. 130-093-924). On day 3, half of the medium was replaced with fresh medium containing supplements. On day 5, iDCs were harvested by collecting non-adherent cells and adherent cells were detached by incubation with Dulbecco's phosphate-buffered saline (DPBS) containing 2 mM EDTA for 10 min at 37°C. After washing with DPBS iDCs were cryopreserved in FBS (Sigma-Aldrich, cat. no. F7524) containing 10% DMSO (AppliChem GmbH, cat. no A3672,0050) for future use in antigen-specific T-cell assays.

One day prior to the start of an antigen-specific $CD8^+$ T-cell stimulation assay, frozen PBLs and iDCs from the same donor were thawed. $CD8^+$ T cells were isolated from PBLs by MACS technology using anti-CD8 MicroBeads (Miltenyi, cat. no. 130-045-201), according to the manufacturer's instructions. About 10 × $10^6$ to 15 × $10^6$ $CD8^+$ T cells were electroporated with each 10 µg of *in vitro* transcribed (IVT)-RNA encoding the alpha and beta chains of a murine TCR specific for human claudin-6 (CLDN6; HLA-A*02-restricted; described in WO 2015150327 A1) in 250 µL X-VIVO™ 15 medium (Lonza, cat. no. BE02-060Q). The cells were transferred to a 4-mm electroporation cuvette (VWR International GmbH, cat. no. 732-0023) and electroporated using the BTX ECM® 830 Electroporation System (BTX; 500 V, 3 ms pulse). Immediately after electroporation, cells were transferred into fresh IMDM GlutaMAX medium (Life Technologies GmbH, cat. no. 319800-030) containing 5% pooled human serum and rested at 37°C, 5% $CO_2$ for at least 1 hour. T cells were labeled using 0.8 µM carboxyfluorescein succinimidyl ester (CFSE; Life Technologies GmbH, cat. No V12883) in PBS according to the manufacturer's instructions and incubated in IMDM medium supplemented with 5% human serum overnight.

Up to 5 × $10^6$ thawed iDCs were electroporated with 2 µg IVT-RNA encoding full-length human CLDN6 (WO 2015150327 A1), in 250 µL X VIVO™ 15 medium, using the electroporation system as described above (300 V, 12 ms pulse) and incubated in IMDM medium supplemented with 5% pooled human serum overnight.

Electroporated iDCs were incubated with electroporated, CFSE-labeled T cells at a ratio of 1:10 (DC:T cells) in the presence of IgG1-CD27-A-P329R-E345R (0.1, 1 or 10 µg/mL), DuoBody-CD40x4-1BB (0.0022, 0.0067 or 0.2 µg/mL), or a combination of both in IMDM

medium containing 5% pooled human serum in a 96-well round-bottom plate. After 4 days of culture, the cells were stained with an APC-conjugated anti-human CD8 antibody (e.g., PE-Cy7-conjugated, BD Biosciences, cat. no. 557750). T-cell proliferation was evaluated by flow cytometry analysis of CFSE dilution in $CD8^+$ T cells using a BD FACSCelesta™ flow cytometer (Becton Dickinson GmbH). Flow cytometry data was analyzed using FlowJo software version 10.7.1. CFSE label dilution of $CD8^+$ T cells was assessed using the proliferation modeling tool in FlowJo, and expansion indices calculated using the following formula:

Number of cells at start of culture = (G0) + (G1)/2 + (G2)/4 + (G3)/8 + (G4)/16 +…(GN/2N)

Expansion index = Total cell number (sum G0 to GN) / Number of cells at start

G0 to GN are single proliferation peaks, with G0 representing the undivided cell fraction and GN the cell fraction that divided N times.

Cytokine concentrations in cell culture supernatants were determined by multiplexed electrochemiluminescence immunoassay (ECLIA) using a V-Plex Proinflammatory Panel 1 (human) assay for the detection of human interferon (IFN)γ (Meso Scale Discovery, cat. No. K15049D) following the manufacturer's protocol.

Single agent treatment with IgG1-CD27-A-P329R-E345R or DuoBody-CD40x4-1BB dose-dependently enhanced CD8+ T-cell proliferation compared to co-cultures without antibody treatment. The combination of IgG1-CD27-A-P329R-E345R and DuoBody-CD40x4-1BB further potentiated single-agent activity (Figure 27). Treatment with a combination of 1 or 10 µg/mL IgG1-CD27-A-P329R-E345R and 0.0067 or 0.2 µg/mL DuoBody-CD40x4-1BB led to higher proliferation than treatment with each compound individually, although the increase compared to single-agent DuoBody-CD40x4-1BB was modest.

Single agent treatment with IgG1-CD27-A-P329R-E345R or DuoBody-CD40x4-1BB dose-dependently enhanced secretion of the proinflammatory cytokine IFNγ compared to co-cultures without antibody treatment (Figure 28). Combination treatment with 1 or 10 µg/mL IgG1-CD27-A-P329R-E345R and 0.0067 µg/mL DuoBody-CD40x4-1BB further potentiated cytokine secretion, although the increase compared to single-agent DuoBody-CD40x4-1BB was modest.

**LEGENDS TO THE FIGURES**

Figure 1 shows CD27 agonist activity of anti-CD27 antibodies and hexamerization-enhanced Fc variants thereof as determined in a CD27 Jurkat Reporter BioAssay. Thaw-and-Use GloResponse NFκB-luc2/CD27 Jurkat reporter cells were incubated for 6h with antibody concentration series (from left to right: 0.04 µg/mL, 0.30 µg/mL, 2.50 µg/mL, and 20 µg/mL) of the indicated antibodies. Luciferase activity, as a read-out for CD27 intracellular signaling, was quantified by determining the luminescence (RLU: relative luminescence units). The following antibodies were included as WT IgG1 and/or variants with an E430G or E345R mutation, as indicated: non-binding anti-HIV-gp120 control antibody comprising the E345R mutation (IgG1-b12-E345R, ctrl), anti-CD27 antibodies IgG1-CD27-A, IgG1-CD27-B, IgG1-CD27-C, IgG1-CD27-D, IgG1-CD27-E, and IgG1-CD27-F, and prior art anti-CD27 benchmark antibodies IgG1-CD27-131A and IgG1-CD27-15.

Figure 2 shows binding of anti-CD27 antibodies to (A,B) human and (C,D) cynomolgus monkey CD27 expressed on (A,C) T cells in PBMCs or (B,D) CD27-transfected HEK293F cells, as determined by flow cytometry. Antibody binding is presented as the median fluorescence intensity (MFI). The anti-HIV-gp120 antibody IgG1-b12-FEAR (ctrl) was included as non-binding negative control antibody.

Figure 3 shows binding of anti-CD27 antibodies IgG1-CD27-A, IgG1-CD27-B, and IgG1-CD27-C to human CD27-A59T variant expressed on HEK293F cells, as determined by flow cytometry. Antibody binding is presented as the median MFI. The anti-HIV-gp120 antibody IgG1-b12-FEAL (ctrl) was included as non-binding negative control antibody.

Figure 4 shows heatmaps of the proliferation of TCR stimulated (A) $CD8^+$ and (B) $CD4^+$ T cells in the presence of 1 µg/mL CD27-specific antibody variants IgG1-CD27-A, -B, or -C harboring the Fc mutations E430R or E345R in combination with the Fc mutations P329R, G237A, or K326A-E33A, as determined by flow cytometry in a CSFE dilution assay. PBMC from four human healthy donors were used as a source of T cells. T-cell proliferation was expressed as the T-cell division index or the percentage of proliferated T cells, that was calculated by gating for the cells that have gone through CFSE dilution ($CFSE^{low\ peaks}$) by using the FlowJo software.

Figure 5 shows the (A-D) percentage of proliferated T cells, (E, F) the expansion index of (A, B) unstimulated or (C-F) TCR stimulated (A, C, E) $CD4^+$ or (B, D, F) $CD8^+$ T cells after incubation of human healthy donor PBMC with IgG1-CD27-A, IgG1-CD27-A-P329R-E345R or prior art anti-CD27 clones IgG1-CD27-131A, IgG1-CD27-CDX1127, and IgG1-CD27-BMS986215, as determined by flow cytometry. The anti-HIV-gp120 antibody variant IgG1-b12-E345R-P329R (ctrl) was included as non-binding negative control antibody. % Proliferated cells were calculated by gating for the cells that have gone through CFSE dilution

($CFSE^{low\ peaks}$). Expansion index identifies the fold increase of cells in the wells and was calculated using the Proliferation Modeling tool in FlowJo version 10. Manual adjustments to the peaks were made where necessary to define the number of the peaks present more consistently.

Figure 6 shows binding of C1q to membrane-bound CD27 antibodies of the invention, as determined by FACS. IgG1-CD27-A variants containing a E430G or E345R hexamerization-enhancing mutation (IgG1-CD27-A-E430G and IgG1-CD27-A-E345R) and the P329R mutation (IgG1-CD27-A-P329R-E345R) were tested for their capacity to bind to C1q. The anti-HIV-gp120 antibody IgG1-b12-F405L (ctrl) was included as non-binding negative control antibody.

Figure 7 shows binding of IgG1-CD27-A-P329R-E345R to human Fc receptors as determined by surface plasmon resonance (SPR). Biacore surface chips were covalently linked with anti-His antibody and coated with recombinant His-tagged Fc receptors (A) FcγRIa, (B) FcγRIIa-H, (C) FcγRIIa-R, (D) FcγRIIb, (E) FcγRIIIa-F, or (F) FcγRIIIa-V. The anti-HIV-gp120 antibody IgG1-b12 (ctrl) was included as a reference. Shown are absolute resonance units as determined by Biacore SPR after background subtraction (no Fc receptor flow-cell).

Figure 8 shows binding of IgG1-CD27-A-P329R-E345R to human (A) $CD4^+$ and (B) $CD8^+$ T-cell subsets in human healthy donor PBMC samples, as determined by flow cytometry. Negative control antibody IgG1-b12-P329R-E345R (ctrl) is an anti-HIV gp120 non-binding isotype control antibody comprising the P329R and E345R mutations. Data presented is the mean MFI +/- SD of duplicate samples.

Figure 9 shows CD27 agonist activity of anti-CD27 antibodies in presence and absence of FcγR-mediated crosslinking, as determined in a reporter assay. A fixed number of NFκB-luc2/CD27 Jurkat reporter cells was cultured with (A-E) IgG1-CD27-A-P329R-E345R or IgG1-CD27-A, (F-J) IgG1-CD27-131A, IgG1-CD27-CDX1127 or IgG1-CD27-BMS986215, in (A,F) absence or (B-J) presence of FcγRIIb-CHO-K1 cells, at a NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1 ratio of (B,G) 1:1, (C,H) 1:1/3, (D,I) 1:1/9, or (E,J) 1:1/27. IgG1-b12-P329R-E345R and IgG1-b12 are anti-HIV gp120 non-binding control antibodies (ctrl). Luminescence was measured as a readout for CD27 activation and presented as relative luminescence units (RLU).

Figure 10 shows the human IgG levels in plasma of SCID mice, after intravenous injection of 25 mg/kg IgG-CD27-A or IgG-CD27-A-P329R-E345R antibodies. Total human IgG plasma concentrations were determined by sandwich ELISA and plotted against time after injection. Data shown are mean plasma concentrations +/- SEM of blood samples per group (n=3 mice).

Figure 11 shows the percentage of viable $CD27^+$ Daudi cells after co-culturing for 4 h with hMDM (E:T = 2:1) in the presence of IgG1-CD27-A-P329R-E345R or wild-type CD20 antibody IgG1-CD20. Daudi cells were labeled with CellTrace™ Violet and cell viability was measured by flow cytometry. Data shown are the mean of duplicates ± SD percentage of viable Daudi cells ($TO\text{-}PRO\text{-}3^-CTV^+CD11b^-$) normalized to the no antibody controls for one donor out of four tested in two experiments.

Figure 12 shows C4d deposition upon incubation of IgG1-CD27-A-P329R-E345R in NHS as determined by ELISA. IgG1-b12-P329R-E345R is an isotype control antibody and IgG1-b12 is a control antibody with a WT Fc domain; IgG1-b12-RGY is a positive control antibody for C4d deposition (hexameric antibody in solution). Shown is mean ± SD of triplicates of one representative experiment out of three performed.

Figure 13 shows the inhibition of CD70 binding on Daudi cells by anti-CD27 antibodies. $CD27^+$ Daudi cells were incubated with 6 µg/mL biotinylated recombinant human CD70 ECD in the presence or absence of 50 µg/mL of the non-binding control antibodies (IgG1-b12-P329E-E345R or IgG1-b12) or CD27 antibodies (IgG1-CD27-A, IgG1-CD27-A-P329R-E345R, IgG1-CD27-CDX1127, IgG1-CD27-BMS986215, or IgG1-CD27-131A). Binding of the biotinylated CD70 fragment to the Daudi cells was detected by flow cytometry using BV421-labeled streptavidin. Data shown are the gMFI ± SD from duplicate wells of one representative experiment out of three performed.

Figure 14 shows expression levels of T-cell activation markers in polyclonally activated $CD4^+$ and $CD8^+$ T cells upon treatment with anti-CD27 antibodies. Human healthy donor PBMC were incubated with 0.1 µg/mL CD3 antibody and 30 µg/mL of IgG1-CD27-A-P329R-E345R, CD27 antibody benchmarks or non-binding control antibody IgG1-b12-P329R-E345R for two or five days. The expression levels of T-cell activation markers HLA-DR, CD69, GITR, CD25, CD107a, and 4-1BB on the surface of (A) $CD4^+$ and (B) $CD8^+$ T cells in antibody-treated samples were quantified by flow cytometry and presented as mean fold change in MFI (± SD) relative to the nonbinding control sample of the same donor. Dotted lines indicate the fold change for cells treated with IgG1-b12-P329R-E345R, which was used as a nonbinding control and set to 1. Data shown are from three donors tested in duplicate in one experiment.

Figure 15 shows percentages of OVA-specific $CD8^+$ T cells in spleen of hCD27-KI mice after immunization with OVA and treatment with anti-CD27 antibodies. hCD27-KI mice were injected s.c. with 5 mg OVA on days 0, 12 and 21, and simultaneously treated i.v. with 30 mg/kg IgG1-CD27-A-P329R-E345R, IgG1-CD27-CDX1127 or non-binding control antibody

IgG1-b12-P329R-E345R. On day 28, mice were euthanized, spleens were resected, and processed as single cell suspensions. Expansion of OVA specific $CD8^+$ T cells was evaluated by flow cytometry. Data shown are the mean of % $OVA^+$ of $CD8^+$ cells ± SD per treatment group (5 mice per group) from one experiment performed.

Figure 16 shows the number of IFNγ-producing splenocytes on day 28 after immunization with OVA and treatment with anti-CD27 antibodies as measured by IFNγ-ELISpot. hCD27-KI mice were injected s.c. with 5 mg OVA on days 0, 12 and 21, and simultaneously treated i.v. with 30 mg/kg IgG1-CD27-A-P329R-E345R, IgG1-CD27-CDX1127, or non-binding control antibody IgG1-b12-P329R-E345R. On day 28, spleens were resected, processed as single cell suspensions and IFNγ-producing splenocytes were detected using IFNγ-ELISpot. Data shown are the mean number of spots per well ± SEM of each treatment group from one experiment performed (5 mice per group).

Figure 17 shows the percentage of activated $CD8^+$ T cells in the spleen of hCD27-KI mice after immunization with OVA and treatment with anti-CD27 antibodies. hCD27-KI mice were injected s.c. with 5 mg OVA on days 0, 12 and 21, and simultaneously treated i.v. with 30 mg/kg IgG1-CD27-A-P329R-E345R, IgG1-CD27-CDX1127, or non-binding control antibody IgG1-b12-P329R-E345R. On day 28, mice were euthanized, spleens were resected, and processed as single cell suspensions. Activation of $CD8^+$ T cells was evaluated in spleen samples by measuring the percentage $PD\text{-}1^+$ of $CD8^+$ cells in spleen by flow cytometry. Data shown are the mean ± SD per treatment group (5 mice per group) from one experiment performed.

Figure 18 shows percentages of effector $CD8^+$ T cells in the spleen of hCD27-KI mice after immunization with OVA and treatment with anti-CD27 antibodies. hCD27-KI mice were injected s.c. with 5 mg OVA on days 0, 12 and 21, and simultaneously treated i.v. with 30 mg/kg IgG1-CD27-A-P329R-E345R, IgG1-CD27-CDX1127, or non-binding control antibody IgG1-b12-P329R-E345R. On day 28 mice, were euthanized, spleens were resected, and processed as single cell suspensions. Expansion of memory T cells was evaluated by expression of CD44 and CD62L by flow cytometry. Data shown are the mean ± SD per treatment group (5 mice per group) from one experiment performed. (A) Percentage $CD8^+CD44^+CD62L^-$ effector memory of $CD45^+$ cells. (B) Percentage $CD44^+CD62L^-$ effector memory of $CD8^+$ T cells. (C) Percentage $CD8^+CD44^-CD62L^-$ pre-effector of $CD45^+$ cells. (D) Percentage $CD44^-CD62L^-$ pre-effector of $CD8^+$ T cells.

Figure 19 shows percentage of T cells in the spleen of hCD27-KI mice after immunization with OVA and treatment with anti-CD27 antibodies. hCD27-KI mice were injected s.c. with 5

mg OVA on days 0, 12 and 21, and simultaneously treated i.v. with 30 mg/kg IgG1-CD27-A-P329R-E345R, IgG1-CD27-CDX1127, or non-binding control antibody IgG1-b12-P329R-E345R. On day 28, mice were euthanized, spleens were resected, and processed as single cell suspensions. $CD3^+$ cells in the blood and spleens were evaluated by flow cytometry. Data shown are the mean ± SD per treatment group (5 mice per group) from one experiment performed.

Figure 20 shows the effect of IgG1-CD27-A-P329R-E345R on T-cell cytokine production in antigen-specific studies. Cocultures of CLDN6-TCR-expressing CD8+ T cells that (A) express endogenous PD-1 or (B) overexpress PD-1 and autologous CLDN6-expressing iDC were incubated with 10 µg/mL IgG1-CD27-A-P329R-E345R, CD27 benchmark antibody IgG1-CD27-131A, or nonbinding control antibody IgG1-b12-P329R-E345R for two days. Cytokine levels in coculture supernatants were analyzed by multiplex ECLIA. Data shown are mean concentrations ± SD of triplicate wells from one representative donor out of seven tested in two experiments performed. Abbreviations: CLDN6 = claudin 6; ECLIA = electrochemiluminescence assay; iDC = immature dendritic cell; PD-1 = programmed cell death protein 1; SD = standard deviation; TCR = T cell receptor.

Figure 21 shows expression of cytotoxicity-associated molecules in antigen-specific CD8+ T cells incubated with IgG1-CD27-A-P329R-E345R. CLDN6-TCR-electroporated CD8+ T cells were cocultured with hCLDN6-MDA-MB-231 cells in the presence of IgG1-CD27-A-P329R-E345R, CD27 benchmark IgG1-CD27-131A, or nonbinding control antibody IgG1-b12-P329R-E345R for two days. Intracellular expression of GzmB and CD107a was determined by flow cytometry. The percentage of CD8+ T cells expressing both GzmB and CD107a, as well as expression levels of GzmB and CD107a (MFI normalized to IgG1-b12-P329R-E345R) in CD8+ T cells is shown. Data shown are mean ± SD of six donors tested in single replicate in experiments two experiments. **, $P<0.01$; *, $P<0.05$; Friedman-test with Dunn's multiple comparisons test. Abbreviations: CLDN6 = claudin 6; GzmB = granzyme B; MFI = mean fluorescence intensity; SD = standard deviation; TCR = T-cell receptor.

Figure 22 shows antigen-specific CD8+ T-cell mediated tumor cell kill in the presence of IgG1-CD27-A-P329R-E345R. CD8+ T-cell mediated kill of hCLDN6-MDA-MB-231 cells was evaluated by real-time cell analysis. CLDN6 TCR electroporated CD8+ T cells were cocultured with hCLDN6-MDA-MB-231 cells in the presence of IgG1-CD27-A-P329R-E345R, CD27 benchmark IgG1-CD27-131A, or nonbinding control antibody IgG1-b12-P329R-E345R for five days. Cell index values were derived from impedance measurements conducted at two-hour intervals. AUC were obtained from cell index data over five days of coculture. The AUC of each

treatment condition was normalized to IgG1-b12-P329R-E345R-treated cultures from the same donor. Data shown are mean ± SD from six donors tested in duplicate in experiments in two experiments. **, P<0.01; Friedman-test with Dunn's multiple comparisons test. Abbreviations: AUC = area under the curve; CLDN6 = claudin 6; SD = standard deviation; TCR = T-cell receptor.

Figure 23 shows absolute cell numbers of CD4+ and CD8+ T cells and NK cells in primary tumor cultures after treatment with IgG1-CD27-A-P329R-E345R. Human NSCLC tumor tissues were cultured with low-dose IL-2 (45 to 50 U/mL) in the presence or absence of 10 µg/mL IgG1-CD27-A-P329R-E345R. Absolute cell counts of the TIL subsets were determined by flow cytometry after 14 days of treatment. Data shown are average ± SD of four replicate wells from one out of five tumor tissues tested in one experiment out of four performed. Abbreviations: IL = interleukin; NK = natural killer; NSCLC = non-small cell lung cancer; SD = standard deviation; U/mL = units per mL.

Figure 24 shows molecular proximity determined by bioluminescence resonance energy transfer (BRET) analysis between IgG1-CD27-A-P329R-E345R antibodies on the cell surface of Daudi and huCD27-K562 cells. Cells were incubated with mixtures of NanoLuc- (donor) and HaloTag- (acceptor) tagged antibodies (5 µg/mL each): IgG1-CD27-A-P329R-E345R, WT IgG1-CD27-A or nonbinding control IgG1-b12-P329R-E345R as indicated. The antibody pair IgG1-CD20-11B8-E430G-LNLuc and IgG1-CD37-37.3-E430G-LHalo was used as positive control. BRET was calculated in milliBRET units (mBU) = (618 $nm_{em}$/460 $nm_{em}$) x 1000, and corrected for donor bleed-through by subtracting no-ligand control values. Data shown are the corrected BRET from duplicate wells of one representative experiment out of three performed.

Figure 25 shows binding of IgG1-CD27-A-P329R-E345R to M0 and M1 macrophages compared to a WT IgG1 antibody (IgG1-b12) with an irrelevant antigen-binding region as a positive control for FcγRIa binding, and a variant of the same antibody carrying the P329R and E345R mutations (IgG1-b12-P329R-E345R). Binding of the antibodies to the macrophages was detected by flow cytometry using PE-labeled goat anti-human secondary antibody. Data shown are mean + SD of two donors tested.

Figure 26 shows proliferation of polyclonally activated CD8+ T cells induced by IgG1-CD27-A-P329R-E345R in combination with DuoBody-CD40x4-1BB. CTV-labeled human healthy donor PBMC were incubated with CD3 antibody and IgG1-CD27-A-P329R-E345R and/or DuoBody-CD40x4-1BB for four days. CTV dilution in T cells was analyzed by flow cytometry and used to calculate the expansion index. Data shown are from CD8+ T cells in samples

stimulated with 0.1 µg/mL CD3 antibody. Values present expansion indices of single replicates from one representative donor out of six donors tested in four experiments performed. Abbreviations: CD = cluster of differentiation; CTV = cell trace violet; PBMC = peripheral blood mononuclear cells; TCR = T-cell receptor.

Figure 27 shows the effect of IgG1-CD27-A-P329R-E345R in combination with DuoBody-CD40x4-1BB on T-cell proliferation in vitro. Human CD8+ T cells were electroporated with RNA encoding a CLDN6-specific TCR and labeled with CFSE. The T cells were then co-cultured with iDCs electroporated with CLDN6-encoding RNA, in the absence or presence of DuoBody-CD40x4-1BB (0.0022, 0.0067 or 0.2 µg/mL), IgG1-CD27-A-P329R-E345R (0.1, 1 or 10 µg/mL), or combinations of both, for 4 d. CFSE dilution in T cells was analyzed by flow cytometry and used to calculate the expansion index. Data from one representative donor out of six donors tested in three independent experiments are shown. Error bars indicate SD of duplicate wells. Dotted line represents expansion index of CD8+ T cells co-cultured with iDCs without antibody treatment. CFSE, Carboxyfluorescein succinimidyl ester; CLDN6, claudin-6; iDC, immature dendritic cell; SD, standard deviation; TCR, T-cell receptor.

Figure 28 shows the effect of IgG1-CD27-A-P329R-E345R in combination with DuoBody-CD40x4-1BB on IFNγ secretion in vitro. Human CD8+ T cells expressing a CLDN6-specific TCR were co-cultured with CLDN6-expressing iDCs as in Figure 3, in the absence or presence of DuoBody-CD40x4-1BB (0.0022, 0.0067 or 0.2 µg/mL), IgG1-CD27-A-P329R-E345R (0.1, 1 or 10 µg/mL), or combinations of both. After 2 d, IFNγ concentrations were determined in the supernatants by multiplexed ECLIA. Data from one representative donor of three donors tested are shown. Error bars indicate SD of duplicate wells. Dotted line represents IFNγ concentration in CD8+ T cell/iDC co-culture without antibody treatment. CLDN6, claudin-6; ECLIA, electrochemiluminescence immunoassay; iDC, immature dendritic cell; SD, standard deviation; TCR, T-cell receptor.

## CLAIMS

1. A method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject
i) a first binding agent comprises at least one binding region binding to CD27; and
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

2. The method of claim 1, wherein said first binding agent comprises a heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and a light chain variable (VL) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10 and 11, respectively.

3. The method of claim 1 or 2, wherein said first binding agent comprises two binding regions capable of binding to human CD27 wherein said first binding agent comprises the heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and the light chain variable (VL) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10, and 11, respectively.

4. The method of any one of the preceding claims, wherein said first binding agent comprises a VH region comprising a sequence as set forth in SEQ ID NO: 4.

5. The method of any one of the preceding claims, wherein said first binding agent comprises a VL region comprising a sequence as set forth in SEQ ID NO: 8.

6. The method of any one of the preceding claims, wherein said first binding agent comprises the VH and VL regions comprising the sequences as set forth in SEQ ID NO: 4 and SEQ ID NO: 8, respectively.

7. The method of any one of the preceding claims, wherein said first binding agent is an antibody, preferably a human or a humanized antibody.

8. The method of any one of the preceding claims, wherein the antibody is a full-length antibody further comprising a light chain constant region (CL) and a heavy chain constant region (CH).

9. The method of claim 8, wherein the light chain constant region is human kappa.

10. The method of claim 8, wherein the light chain constant region is human lambda.

11. The method of any one of the preceding claims, wherein said first binding agent further comprises a heavy chain constant region, which is of a human IgG isotype, optionally of a modified human IgG.

12. The method of claim 11, wherein the human IgG or modified human IgG is selected from IgG1, IgG2, IgG3 or IgG4, such as human IgG1.

13. The method of claim 11 or 12, wherein the IgG is a modified human IgG comprising one or more amino acid substitutions.

14. The method of any one of claims 11 to 13, wherein the modified human IgG is a modified human IgG1 comprising one or more amino acid substitutions, such as two or more amino acid substitutions.

15. The method of any one of claims 11 to 14, wherein the modified human IgG heavy chain constant region comprises at most 10 amino acid substitutions, such as at most 9, such as at most 8, such as at most 7, such as at most 6, such as at most 5, such as at most 4, such as at most 3, such as at most 2 amino acid substitutions.

16. The method of any one of claims 11 to 15, wherein said substitution in the heavy chain constant region induces increased CD27 agonism compared to an identical antibody except for comprising a wild type IgG1 antibody heavy chain constant region.

17. The method of any one of claims 11 to 16, wherein the amino acid residue at the position corresponding to position E345 or E430 in a human IgG1 heavy chain according to Eu numbering is selected from the group comprising: A, C, D, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y.

18. The method of any one of claims 11 to 17, wherein the amino acid residue at the position corresponding to position E345 in a human IgG1 heavy chain according to Eu numbering is R.

19. The method of any one of claims 11 to 18, wherein the amino acid residue at the position corresponding to position E430 in a human IgG1 heavy chain according to Eu numbering is G.

20. The method of any one of claims 11 to 19, wherein the amino acid residue at the position corresponding to position P329 in a human IgG1 heavy chain according to Eu numbering is R.

21. The method of any one of claims 11 to 20, wherein the amino acid residue at the positions corresponding to position E345 and P329 in a human IgG1 heavy chain according to Eu numbering are both R.

22. The method of any one of claims 11 to 21, wherein the first binding agent has a pharmacokinetic profile as the parent antibody comprising a wild type IgG1 heavy chain constant region.

23. The method of any one of the preceding claims, wherein the first binding agent comprises the heavy chain constant region comprising a sequence selected from the group comprising: SEQ ID No 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 27, 28, 29, 30, 31, 32, 33, 34 and 36.

24. The method of any one of the preceding claims, wherein the first binding agent comprises the heavy chain constant region comprising the sequence as set forth in SEQ ID No 15.

25. The method of any one of the preceding claims, wherein said first binding agent comprises a heavy chain constant region, which is modified so that the first binding agent induces one or more Fc-mediated effector functions to a lesser extent relative to a parent antibody.

26. The method of claim 25, wherein the one or more Fc-mediated effector functions is decreased by at least 20%, such as by at least 30% or by at least 40%, or by at least 50% or by at least 60% or by at least 70%, or by at least 80% or by at least 90%.

27. The method of claim 25 or 26, wherein the first binding agent does not induce one or more Fc-mediated effector functions.

28. The method of any one of claims 25 to 27, wherein the one or more Fc-mediated effector functions is selected from the following group: complement-dependent cytotoxicity (CDC), complement-dependent cell-mediated cytotoxicity (CDCC), complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), C1q binding and FcγR binding.

29. The method of any one of claims 25 to 28, wherein the first binding agent does not induce C1q binding when measured by the method of Example 8.

30. The method of any one of the preceding claims, wherein the first binding agent is a monovalent antibody.

31. The method of any one of the preceding claims, wherein the first binding agent is a bivalent antibody.

32. The method of any one of the preceding claims, wherein the first binding agent is a monospecific antibody.

33. The method of any one of the preceding claims, wherein the first binding agent is a bispecific antibody comprising a first antigen binding region capable of binding human CD27 according to any one of the preceding claims and comprising a second antigen binding region capable of binding to a different epitope on human CD27 or capable of binding a different target.

34. The method of any one of the preceding claims, wherein CD27 is human CD27, in particular said human CD27 comprises the sequence as set forth in SEQ ID NO: 1 or the human CD27 variant as set forth in SEQ ID NO: 2.

35. The method of any one of the preceding claims, wherein said first binding agent comprises:

e. The VH region comprising the amino acid sequence set forth in SEQ ID No: 4;

f. The VL region comprising the amino acid sequence set forth in SEQ ID No: 8;

g. The CH region comprising the amino acid sequence set forth in SEQ ID No: 15; and

h. The CL region comprising the amino acid sequence set forth in SEQ ID No: 17.

36. The method of any one of the preceding claims, wherein said first binding agent comprises a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 35 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 25.

37. The method of any one of the preceding claims, wherein the first binding agent is in a composition or formulation comprising acetate, sorbitol, polysorbate 80, and has a pH from 5 to 6, preferably 5.5.

38. The method of any one of the preceding claims, wherein CD40 is human CD40, in particular human CD40 comprising the sequence set forth in SEQ ID NO: 62, and/or CD137 is human CD137, in particular human CD137 comprising the sequence set forth in SEQ ID NO: 63.

39. The method of any one of the preceding claims, wherein a) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 44, 45, and 46, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 47, YTS , and SEQ ID NO: 48, respectively; and b) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 51, 52, and 53, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 54, GAS, and SEQ ID NO: 55, respectively.

40. The method of any one of the preceding claims, wherein

a) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 49 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 50; and

b) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 56 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 57.

41. The method of any one of the preceding claims, wherein the second binding agent is a multispecific antibody, such as a bispecific antibody.

42. The method of any one of the preceding claims, wherein the second binding agent is in the format of a full-length antibody or an antibody fragment.

43. The method of any one of the preceding claims, wherein the second binding agent is an antibody comprising a first binding arm and a second binding arm, wherein
the first binding arm comprises
i) a polypeptide comprising said first heavy chain variable region (VH) and a first heavy chain constant region (CH), and
ii) a polypeptide comprising said first light chain variable region (VL) and a first light chain constant region (CL);
and the second binding arm comprises
iii) a polypeptide comprising said second heavy chain variable region (VH) and a second heavy chain constant region (CH), and
iv) a polypeptide comprising said second light chain variable region (VL) and a second light chain constant region (CL).

44. The method of any one of the preceding claims, wherein said second binding agent comprises
i) a first heavy chain and light chain comprising said first binding region capable of binding to CD40, the first heavy chain comprising a first heavy chain constant region and the first light chain comprising a first light chain constant region; and
ii) a second heavy chain and light chain comprising said second binding region capable of binding CD137, the second heavy chain comprising a second heavy chain constant region and the second light chain comprising a second light chain constant region.

45. The method of claim 43 or 44, wherein (i) the amino acid in the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering is L in said first heavy chain

constant region (CH), and the amino acid in the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering is R in said second heavy chain constant region (CH), or (ii) the amino acid in the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering is R in said first heavy chain, and the amino acid in the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering is L in said second heavy chain.

46. The method of any one of claim 43-45, wherein the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to EU numbering are F and E, respectively, in said first and second heavy chains.

47. The method of any one of claim 43-46, wherein the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain according to EU numbering are F, E, and A, respectively, in said first and second heavy chain constant regions (HCs).

48. The method of any one of claims 43-47, wherein the positions corresponding to positions L234 and L235 in a human IgG1 heavy chain according to EU numbering of both the first and second heavy chain constant regions are F and E, respectively, and wherein (i) the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is L, and the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is R, or (ii) the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is R, and the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is L.

49. The method of any one of claims 43-48, wherein the positions corresponding to positions L234, L235, and D265 in a human IgG1 heavy chain according to EU numbering of both the first and second heavy chain constant regions are F, E, and A, respectively, and wherein (i) the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the first heavy chain constant region is L, and the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the second heavy chain constant region is R, or (ii) the position corresponding to K409 in a human IgG1 heavy chain according to EU numbering of the first heavy chain is R, and the position corresponding to F405 in a human IgG1 heavy chain according to EU numbering of the second heavy chain is L.

50. The method of any one of claims 43-49, wherein the constant region of said first and/or second heavy chain, such as the second heavy chain, comprises or consists essentially of or consists of an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 58 or 60 [IgG1-Fc_FEAL];

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 6 substitutions, such as at most 5 substitutions, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

51. The method of any one of claims 43-50, wherein the constant region of said first and/or second heavy chain, such as the first heavy chain, comprises or consists essentially of or consists of an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 59 or 61 [IgG1-Fc_FEAR];

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 6 substitutions, such as at most 5 substitutions, at most 4, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

52. The method of any one of any one of claims 43-51, wherein said second binding agent comprises a kappa (κ) light chain constant region.

53. The method of any one of any one of claims 43-52, wherein said second binding agent comprises a lambda (λ) light chain constant region.

54. The method of any one of any one of claims 43-53, wherein said first light chain constant region is a kappa (κ) light chain constant region or a lambda (λ) light chain constant region.

55. The method of any one of any one of claims 43-54, wherein said second light chain constant region is a lambda (λ) light chain constant region or a kappa (κ) light chain constant region.

56. The method of any one of any one of claims 43-55, wherein said first light chain constant region is a kappa (κ) light chain constant region and said second light chain constant region is a lambda (λ) light chain constant region or said first light chain constant region is a lambda (λ) light chain constant region and said second light chain constant region is a kappa (κ) light chain constant region.

57. The method of any one of claims 52-56, wherein the kappa (κ) light chain comprises an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 16,

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 10 substitutions, such as at most 9 substitutions, at most 8, at most 7, at most 6, at most 5, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

58. The method of any one of claims 53-57, wherein the lambda (λ) light chain comprises an amino acid sequence selected from the group consisting of

a) the sequence set forth in SEQ ID NO: 17,

b) a subsequence of the sequence in a), such as a subsequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 consecutive amino acids has/have been deleted, starting from the N-terminus or C-terminus of the sequence defined in a); and

c) a sequence having at most 10 substitutions, such as at most 9 substitutions, at most 8, at most 7, at most 6, at most 5, at most 4 substitutions, at most 3, at most 2 substitutions or at most 1 substitution, compared to the amino acid sequence defined in a) or b).

59. The method of any one of the preceding claims, wherein the second binding agent is of an isotype selected from the group consisting of IgG1, IgG2, IgG3, and IgG4.

60. The method of any one of the preceding claims, wherein the second binding agent is a full-length IgG1 antibody.

61. The method of any one of the preceding claims, wherein the second binding agent is an antibody of the IgG1m(f) allotype.

62. The method of an one of the preceding claims wherein the second binding agent is a bispecific antibody binding to CD40 and CD137, the bispecific antibody having i) a first heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 64 and a first light chain comprising the amino acid sequence set forth in SEQ ID NO: 65 , and ii) a second heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 66 and a second light chain comprising the amino acid sequence set forth in SEQ ID NO: 67.

63. The method of any one of the preceding claims, wherein

a) the first binding agent comprises a heavy chain variable (VH) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NOs: 5, 6, and 7, respectively, and a light chain variable (VL) region CDR1, CDR2, and CDR3 comprising the sequences as set forth in SEQ ID NO: 9, 10, and 11, respectively;

b) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 44, 45, and 46, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 47, YTS, and SEQ ID NO: 48, respectively; and

c) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 51, 52, and 53, respectively, and a light chain variable region (VL) comprising the CDR1, CDR2, and CDR3 sequences set forth in: SEQ ID NO: 54, GAS, and SEQ ID NO: 55, respectively.

64. The method of any one of the preceding claims, wherein

a) the first binding agent comprises a VH region comprising the amino acid sequence set forth in SEQ ID No: 4, a VL region comprising the amino acid sequence set forth in SEQ ID No: 8;

b) the first binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 49 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 50; and

c) the second binding region of the second binding agent comprises a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 56 and a light chain variable region (VL) region comprising the amino acid sequence set forth in SEQ ID NO: 57.

65. The method of any one of the preceding claims, wherein

a) said first binding agent is an antibody comprising a VH region comprising the amino acid sequence set forth in SEQ ID No: 4, a VL region comprising the amino acid sequence set forth in SEQ ID No: 8, a CH region comprising the amino acid sequence set forth in SEQ ID No: 15, and a CL region comprising the amino acid sequence set forth in SEQ ID No: 17;

b) said second binding agent is an antibody comprising a first binding arm and a second binding arm, the first binding arm comprising the first binding region and a second binding arm comprising the second binding region;

c) the first binding arm of the second binding agent comprises a VH region comprising the amino acid sequence set forth in SEQ ID No: 49, a VL region comprising the amino acid sequence set forth in SEQ ID No: 50; a CH region comprising the amino acid sequence set forth in SEQ ID No: 60, and a CL region comprising the amino acid sequence set forth in SEQ ID No: 16; and

d) the second binding arm of the second binding agent comprises a VH region comprising the amino acid sequence set forth in SEQ ID No: 56, a VL region comprising the amino acid sequence set forth in SEQ ID No: 57, a CH region comprising the amino acid sequence set forth in SEQ ID No: 61, and a CL region comprising the amino acid sequence set forth in SEQ ID No: 16.

66. The method of any one of the preceding claims, wherein

c) said first binding agent comprises a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 35 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 25;

d) said second binding agent is a bispecific antibody binding to CD40 and CD137, the bispecific antibody having i) a first heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 64 and a first light chain comprising the amino acid sequence set forth in SEQ ID NO: 65, and ii) a second heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 66 and a second light chain comprising the amino acid sequence set forth in SEQ ID NO: 67.

67. The method of any one of the preceding claims, wherein the subject is a human subject.

68. The method of any one of the preceding claims, wherein the tumor or cancer is a solid tumor.

69. The method according to any one of the preceding claims, wherein said tumor is a PD-L1 positive tumor.

70. The method of any one of the preceding claims, wherein the tumor or cancer is head and neck squamous cell carcinoma (HNSCC), such as HNSCC of the oral cavity, pharynx or larynx.

71. The method of claim 70, wherein the HNSCC is recurrent, unresectable or metastatic.

72. The method of any one of the claims 1-69, wherein the tumor or cancer is non-small cell lung cancer (NSCLC), such as a squamous or non-squamous NSCLC.

73. The method of claim 72, wherein the NSCLC is recurrent, unresectable or metastatic.

74. The method of claim 72 or 73, wherein the NSCLC does not have an epidermal growth factor (EGFR)-sensitizing mutation and/or anaplastic lymphoma (ALK) translocation and/or ROS1 rearrangement.

75. The method of any one of claims 72-74, wherein the NSCLC is NTRK1/2/3 (neurotrophic receptor tyrosine kinase 1/2/3) fusion positive, and/or has a mutation in KRAS (KRAS proto-oncogene, GTPase), BRAF (B-Raf proto-oncogene, serine/threonine kinase), or MET (MET proto-oncogene, receptor tyrosine kinase) gene, and/or has RET (ret proto-oncogene) gene rearrangements, and the subject has received prior treatment with a respective targeted therapy.

76. The method of any one of the preceding claims, wherein the subject has received prior treatment with a PD-1 inhibitor or a PD-L1 inhibitor, such as anti-PD-1 antibody or an anti-PD-L1 antibody, preferably at least two doses of the PD-1 inhibitor or the PD-L1 inhibitor.

77. The method of any one of the preceding claims, wherein the subject has received prior treatment with a platinum-based therapy or an alternative chemotherapy if platinum ineligible, eg a gemcitabine-containing regimen.

78. The method of any one of the preceding claims, wherein the tumor or cancer has relapsed and/or progressed after treatment, such as systemic treatment with a checkpoint inhibitor.

79. The method of any one of the preceding claims, wherein the subject has received at least one prior line of systemic therapy, such as systemic therapy comprising a PD-1 inhibitor or a PD-L1 inhibitor, such as an anti-PD-1 antibody or an anti-PD-L1 antibody.

80. The method of any one of the preceding claims, wherein the cancer or tumor has relapsed and/or is refractory, or the subject has progressed after treatment with a PD-1 inhibitor or a PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody, the PD-1 inhibitor or PD-L1 inhibitor being administered as monotherapy or as part of a combination therapy.

81. The method of any one of the preceding claims, wherein last prior treatment was with a PD1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody, the PD-1 inhibitor or PD-L1 inhibitor being administered as monotherapy or as part of a combination therapy.

82. The method of any one of the preceding claims, wherein the time from progression on last treatment with a PD-1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody is 6 months or less.

83. The method of any one of the preceding claims, wherein the time from last dosing of a PD-1 inhibitor or PD-L1 inhibitor, such as an anti PD-1 antibody or an anti-PD-L1 antibody as part of last prior treatment is 6 months or less.

84. The method of any one of the preceding claims, wherein the cancer or tumor has relapsed and/or is refractory, or the subject has progressed during or after

i) platinum doublet chemotherapy following treatment with an anti-PD-1 antibody or an anti-PD-L1 antibody, or

ii) treatment with an anti-PD-1 antibody or an anti-PD-L1 antibody following platinum doublet chemotherapy.

85. A kit comprising
i) a first binding agent comprising at least one binding region binding to CD27 and
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

86. The kit according to claim 85, wherein the first binding agent is as defined in any one of claims 1-84 and/or the second binding agent is as defined in any one of claims 1-84.

87. The kit according to claim 85 or 86, wherein the first binding agent, the second binding agent, and, if present, one or more additional therapeutic agents are for systemic administration, in particular for injection or infusion, such as intravenous injection or infusion.

88. The kit according to any one of claims 85-87 for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject.

89. The kit for use according to claim 88, wherein the tumor or cancer is as defined in any one of claims 1-84, and/or the subject is as defined in any one of claims 1-84, and/or the method is as defined in any one of claims 1-84.

90. A pharmaceutical composition comprising
i) a first binding agent comprising at least one binding region binding to CD27;
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137; and
iii) optionally a pharmaceutical acceptable carrier.

91. The pharmaceutical composition according to claim 90, wherein the first binding agent is as defined in any one of claims 1-84 and/or the second binding agent is as defined in any one of claims 1-84.

92. The pharmaceutical composition according to claim 90 or 91 for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject.

93. The pharmaceutical composition for use according to claim 92, wherein the tumor or cancer is as defined in any one of claims 1-84, and/or the subject is as defined in any one of claims 1-84, and/or the method is as defined in any one of claims 1-84.

94. A first binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject
i) the first binding agent comprising at least one binding region binding to CD27; and
ii) a second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

95. The first binding agent for use according to claim 94, wherein the method is as defined in any one of claims 1-84, and/or the first binding agent is as defined in any one of claims 1-84, and/or the second binding agent is as defined in any one of claims 1-84.

96. A second binding agent for use in a method for reducing progression or preventing progression of a tumor or treating cancer in a subject, said method comprising administering to said subject
i) a first binding agent comprising at least one binding region binding to CD27; and
ii) the second binding agent comprises a first binding region binding to CD40 and a second binding region binding to CD137.

97. The second binding agent for use according to claim 96, wherein the method is as defined in any one of claims 1-84, and/or the first binding agent is as defined in any one of claims 1-84, and/or the second binding agent is as defined in any one of claims 1-84.

Figure 1

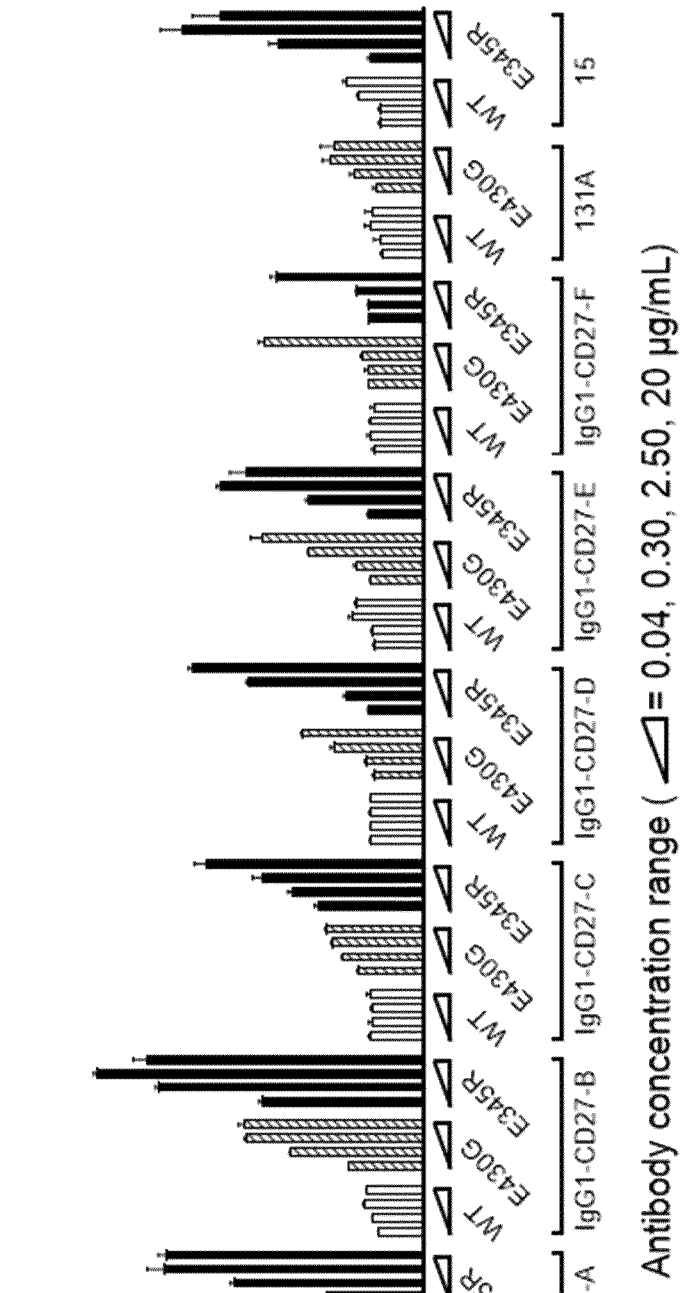

RLU
2,000,000
1,500,000
1,000,000
500,000
0
ctrl
WT
E430G
E345R
IgG1-CD27-A
IgG1-CD27-B
IgG1-CD27-C
IgG1-CD27-D
IgG1-CD27-E
IgG1-CD27-F
131A
15
Antibody concentration range ( = 0.04, 0.30, 2.50, 20 µg/mL)

Figure 2

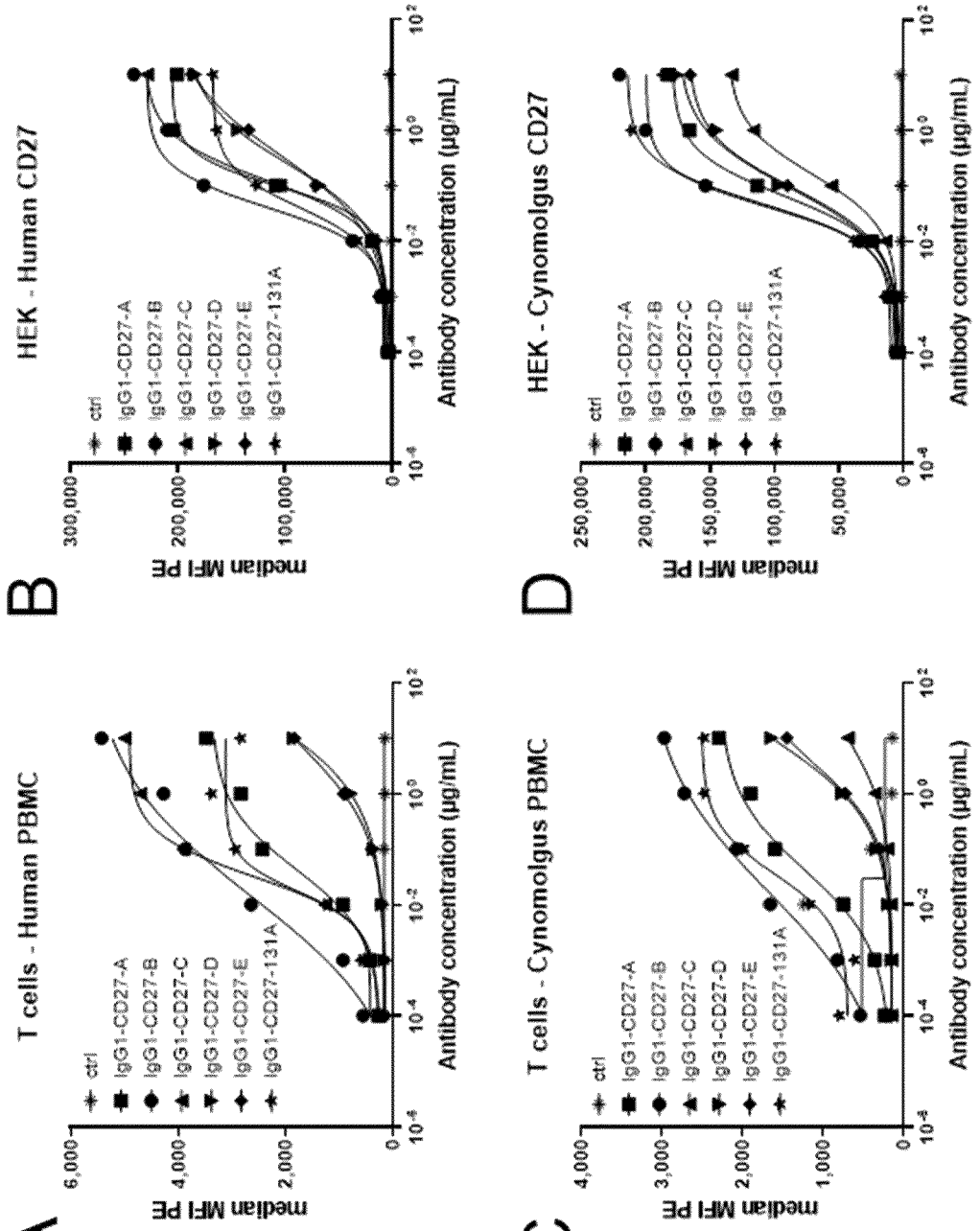

A
T cells - Human PBMC
B
HEK - Human CD27
C
T cells - Cynomolgus PBMC
D
HEK - Cynomolgus CD27
median MFI PE
Antibody concentration (µg/mL)
ctrl
IgG1-CD27-A
IgG1-CD27-B
IgG1-CD27-C
IgG1-CD27-D
IgG1-CD27-E
IgG1-CD27-131A

**Figure 3**

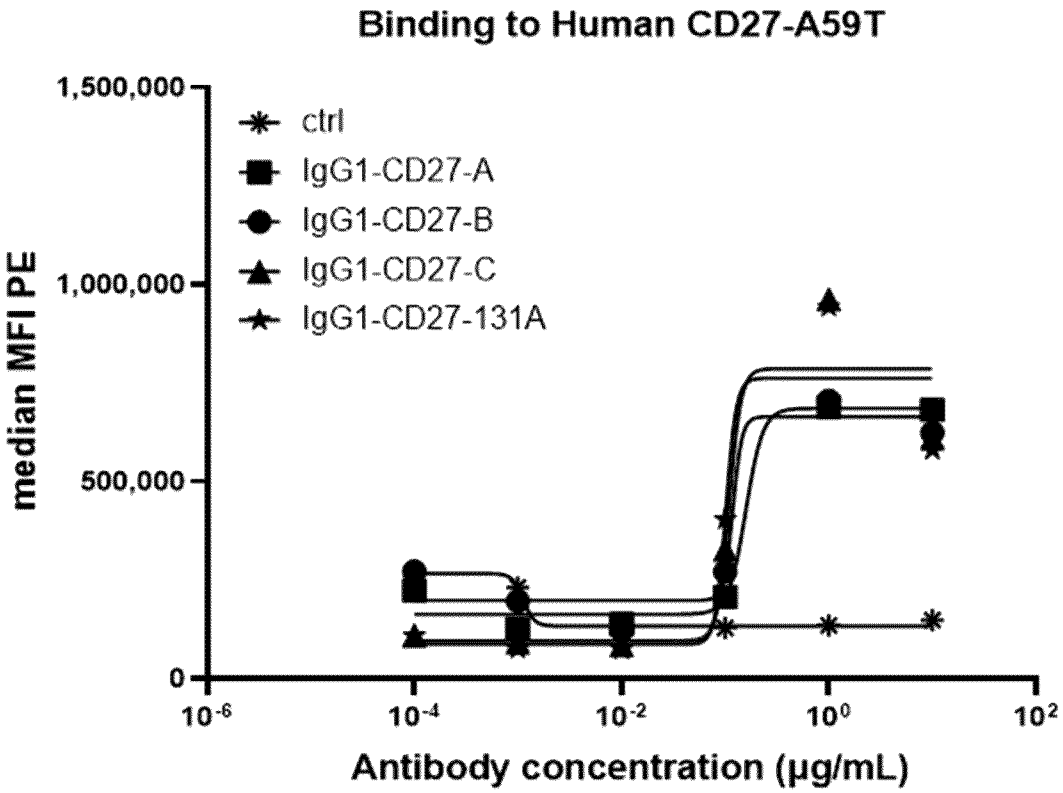
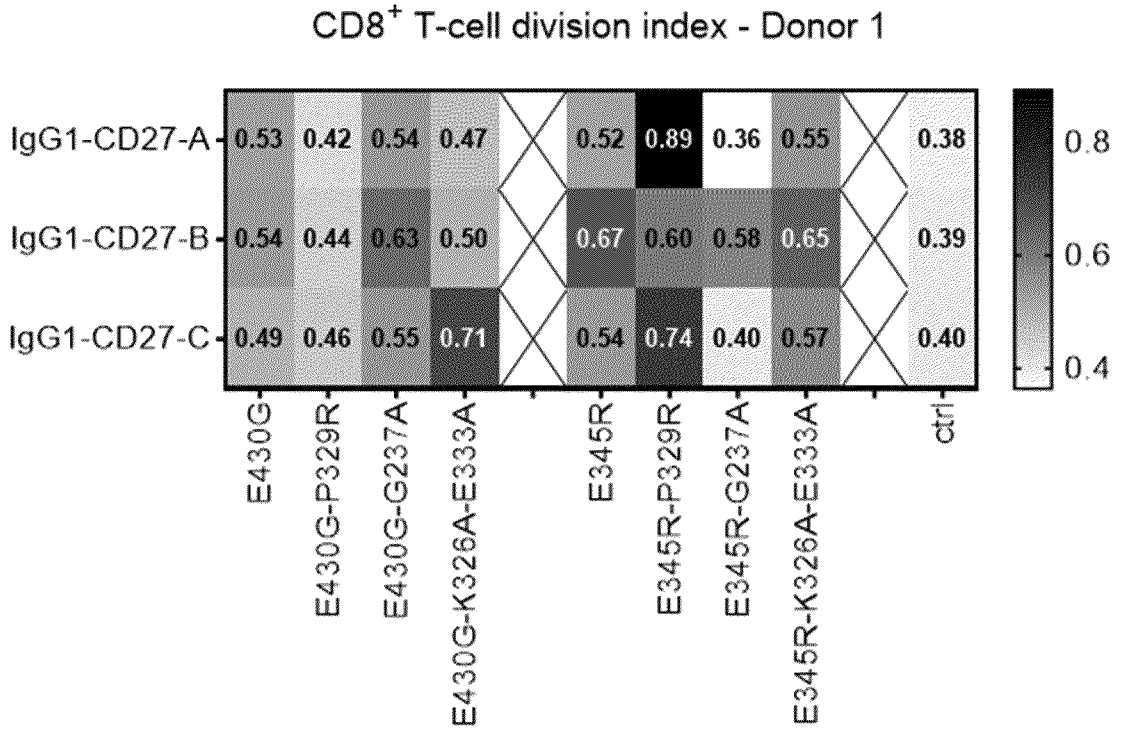

Binding to Human CD27-A59T
1,500,000
1,000,000
500,000
0
median MFI PE
ctrl
IgG1-CD27-A
IgG1-CD27-B
IgG1-CD27-C
IgG1-CD27-131A
10-6
10-4
10-2
100
102
Antibody concentration (µg/mL)

**Figure 4A**

**A: CD8+ T cells, 4 different donors (continued on next page)**



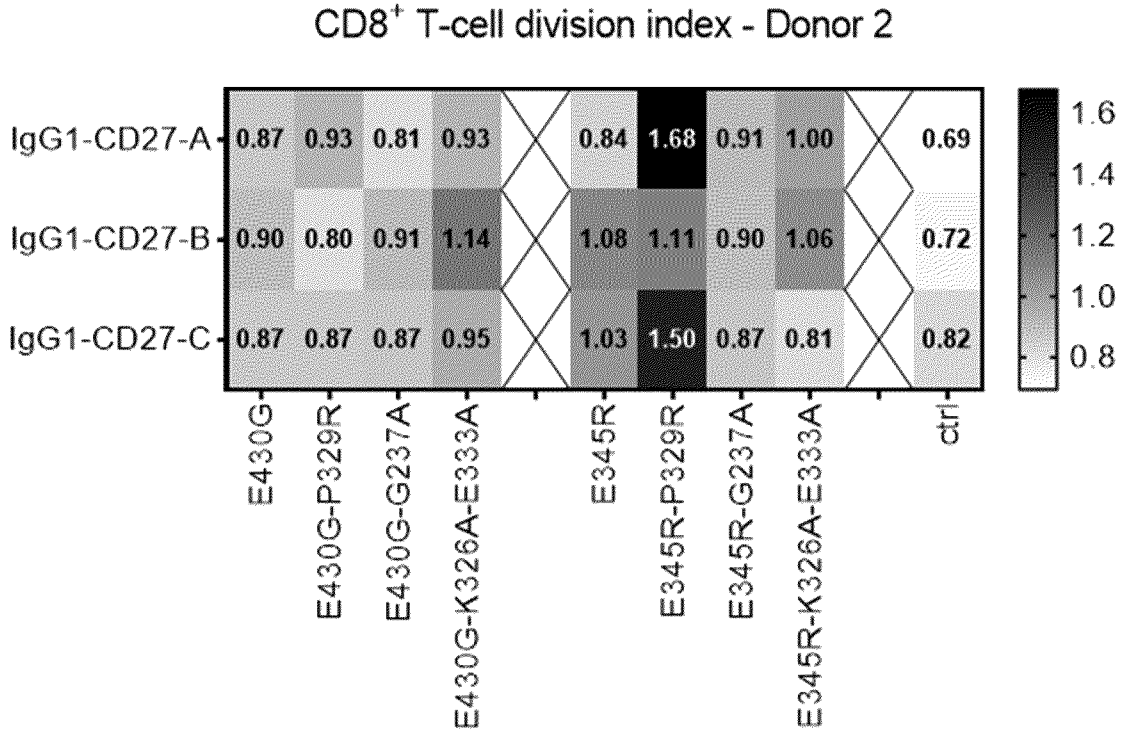
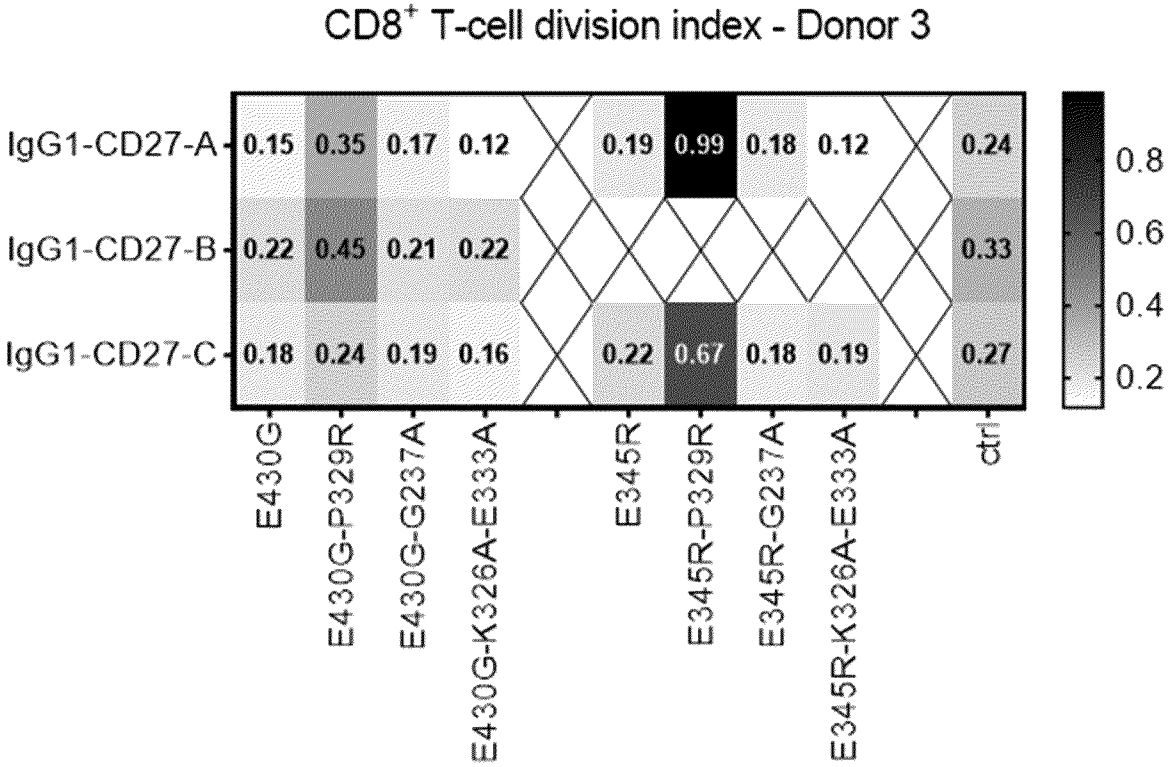



**Figure 4A (cont.)**



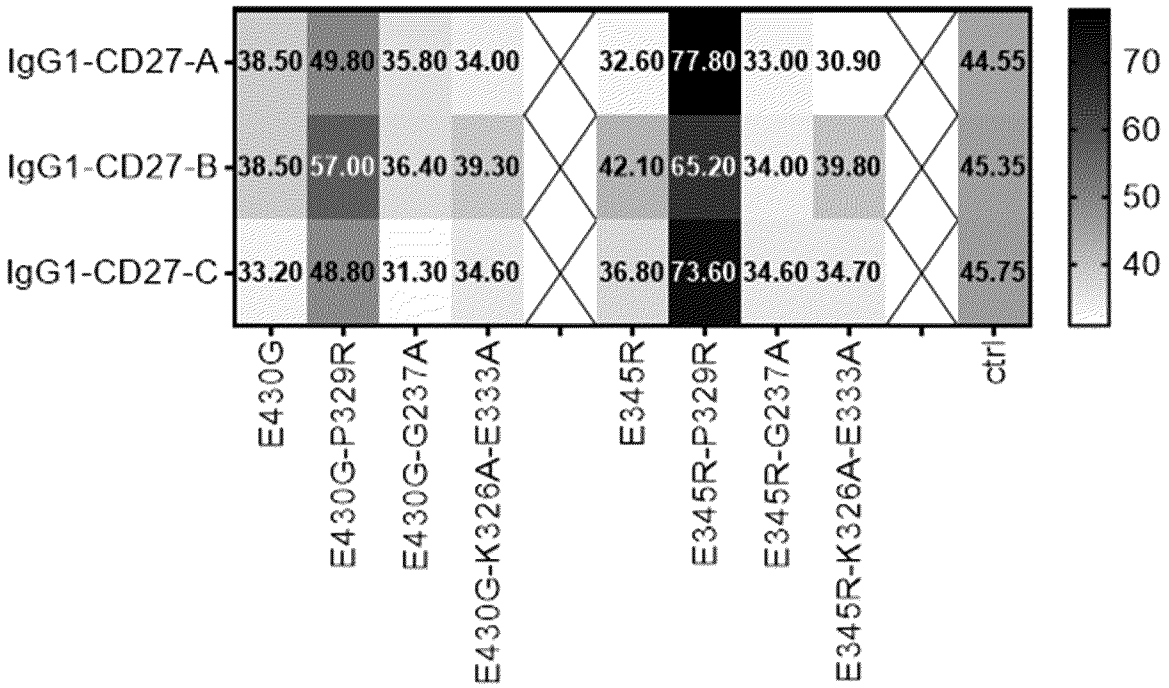



**Figure 4B**

**CD4+ T cells, 4 different donors (continued on next page)**

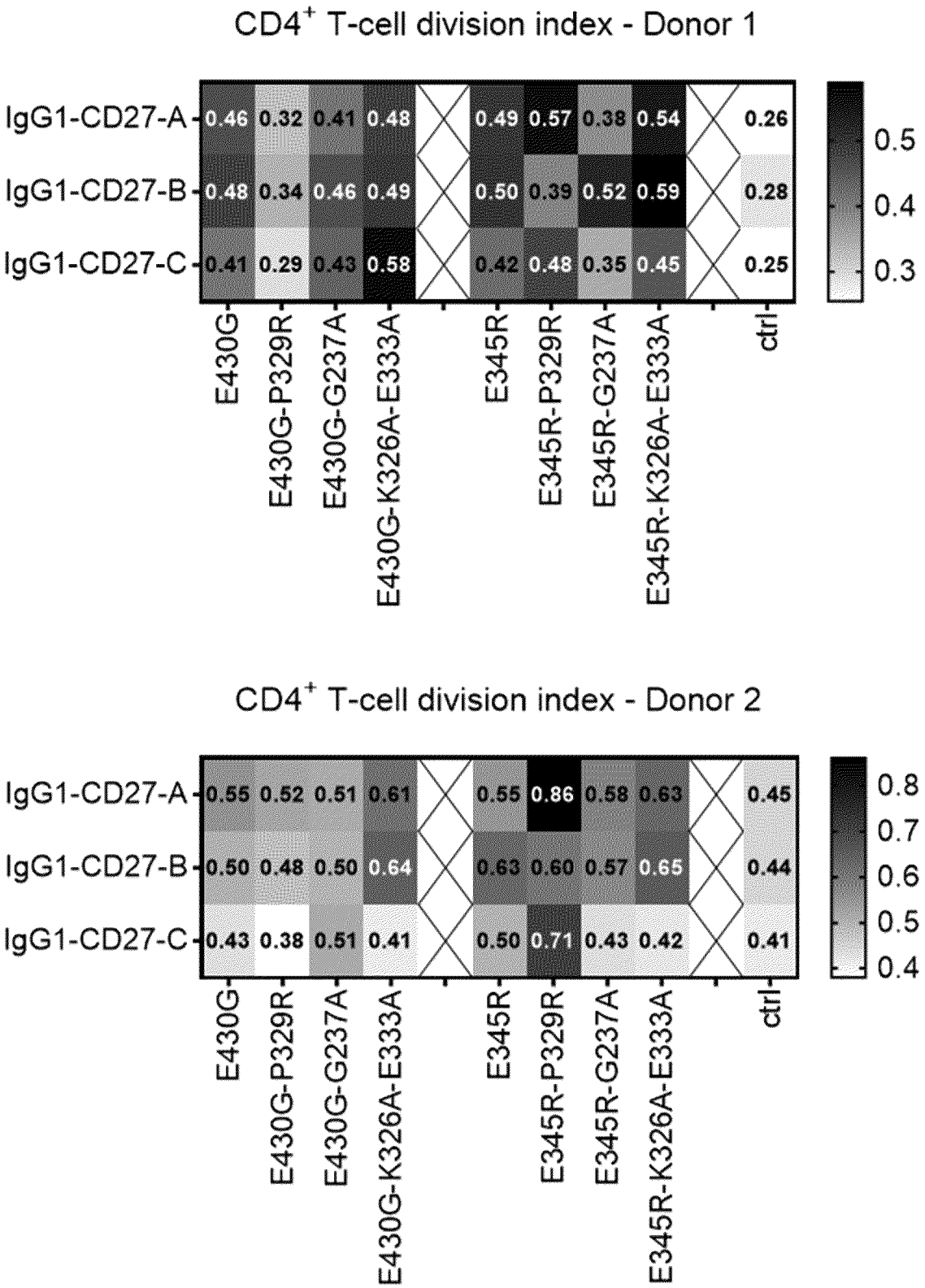


**Figure 4B (cont.)**

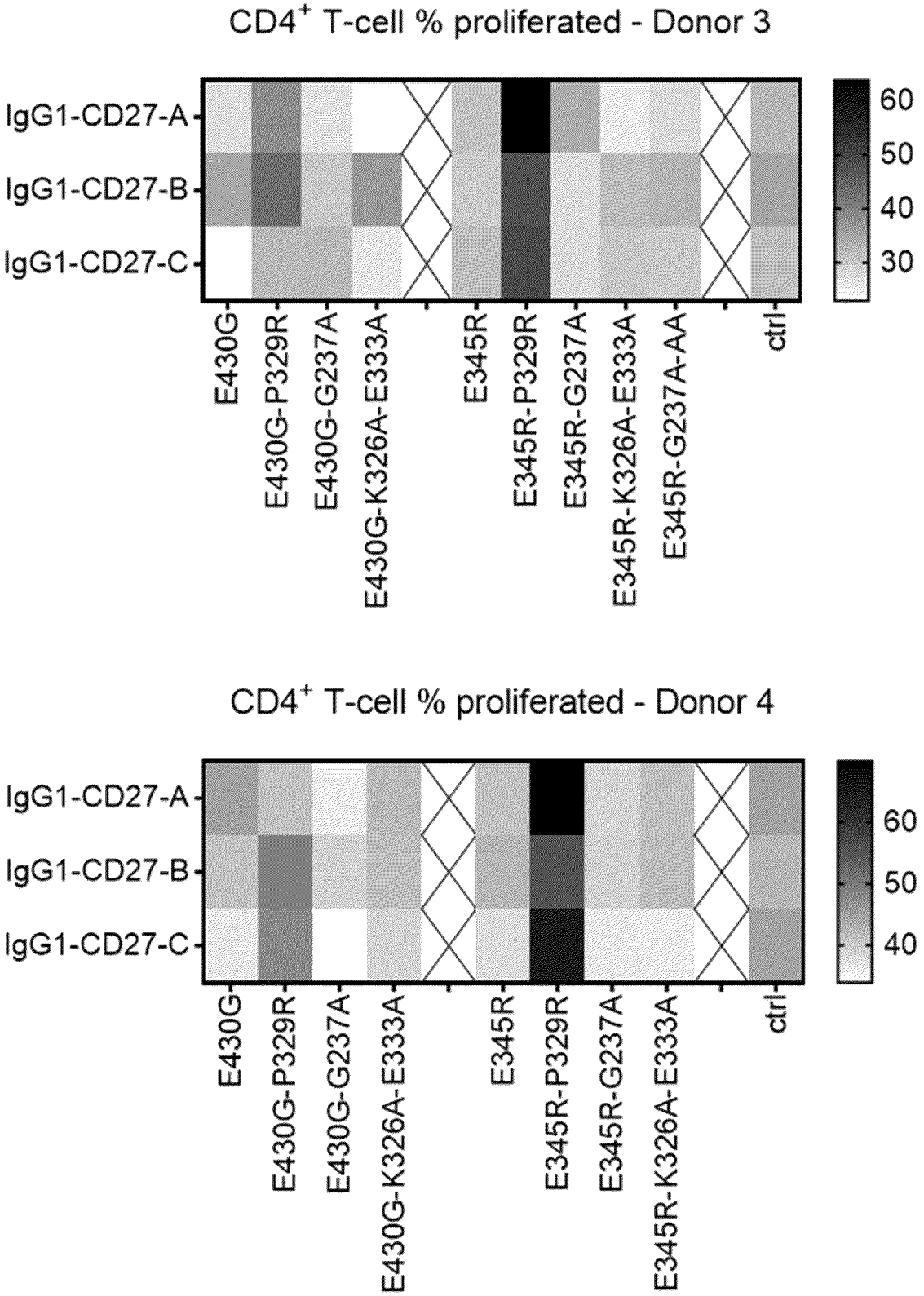


**Figure 5**

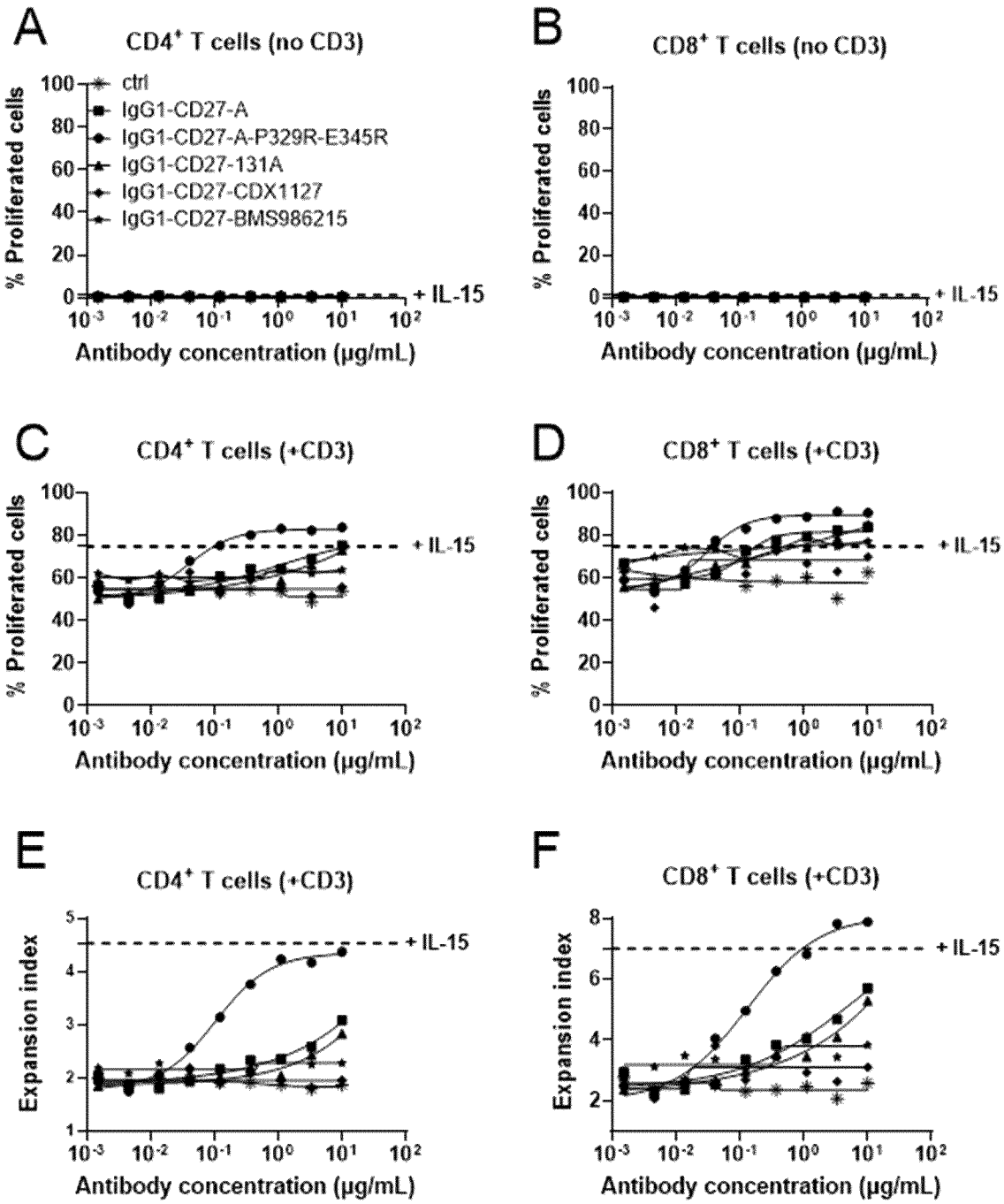

A
CD4+ T cells (no CD3)
% Proliferated cells
ctrl
IgG1-CD27-A
IgG1-CD27-A-P329R-E345R
IgG1-CD27-131A
IgG1-CD27-CDX1127
IgG1-CD27-BMS986215
+ IL-15
Antibody concentration (µg/mL)
B
CD8+ T cells (no CD3)
% Proliferated cells
+ IL-15
Antibody concentration (µg/mL)
C
CD4+ T cells (+CD3)
% Proliferated cells
+ IL-15
Antibody concentration (µg/mL)
D
CD8+ T cells (+CD3)
% Proliferated cells
+ IL-15
Antibody concentration (µg/mL)
E
CD4+ T cells (+CD3)
Expansion index
+ IL-15
Antibody concentration (µg/mL)
F
CD8+ T cells (+CD3)
Expansion index
+ IL-15
Antibody concentration (µg/mL)

**Figure 6**

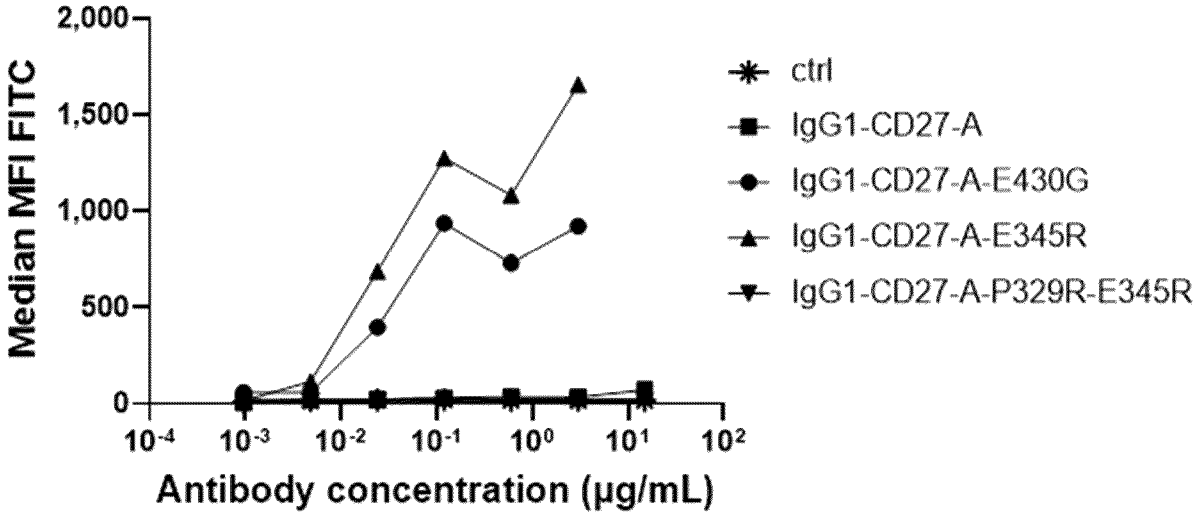

2,000
1,500
1,000
500
0
Median MFI FITC
10-4
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/mL)
ctrl
IgG1-CD27-A
IgG1-CD27-A-E430G
IgG1-CD27-A-E345R
IgG1-CD27-A-P329R-E345R

**Figure 7**

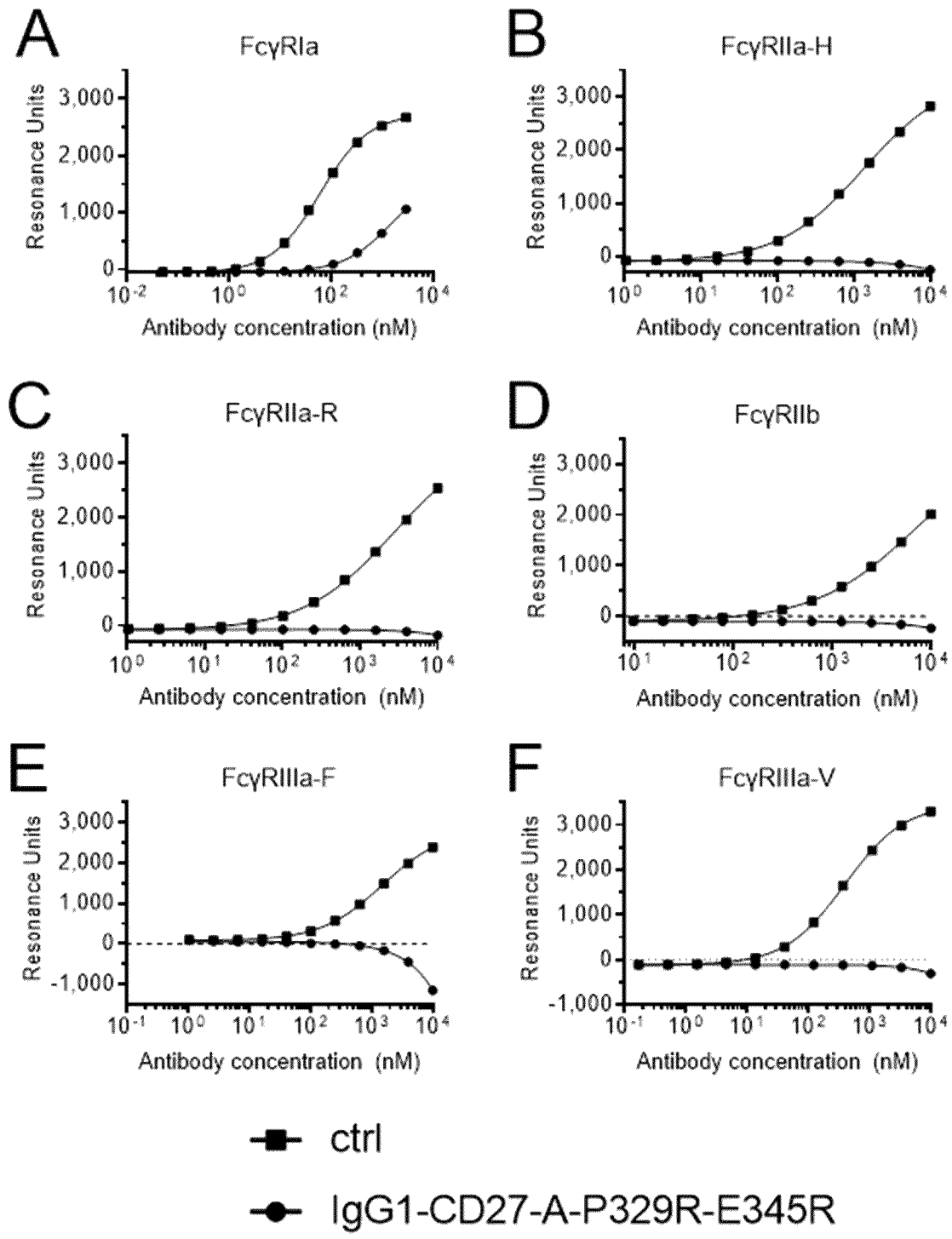

A
FcγRIa
B
FcγRIIa-H
C
FcγRIIa-R
D
FcγRIIb
E
FcγRIIIa-F
F
FcγRIIIa-V
Resonance Units
Antibody concentration (nM)
ctrl
IgG1-CD27-A-P329R-E345R

Figure 8

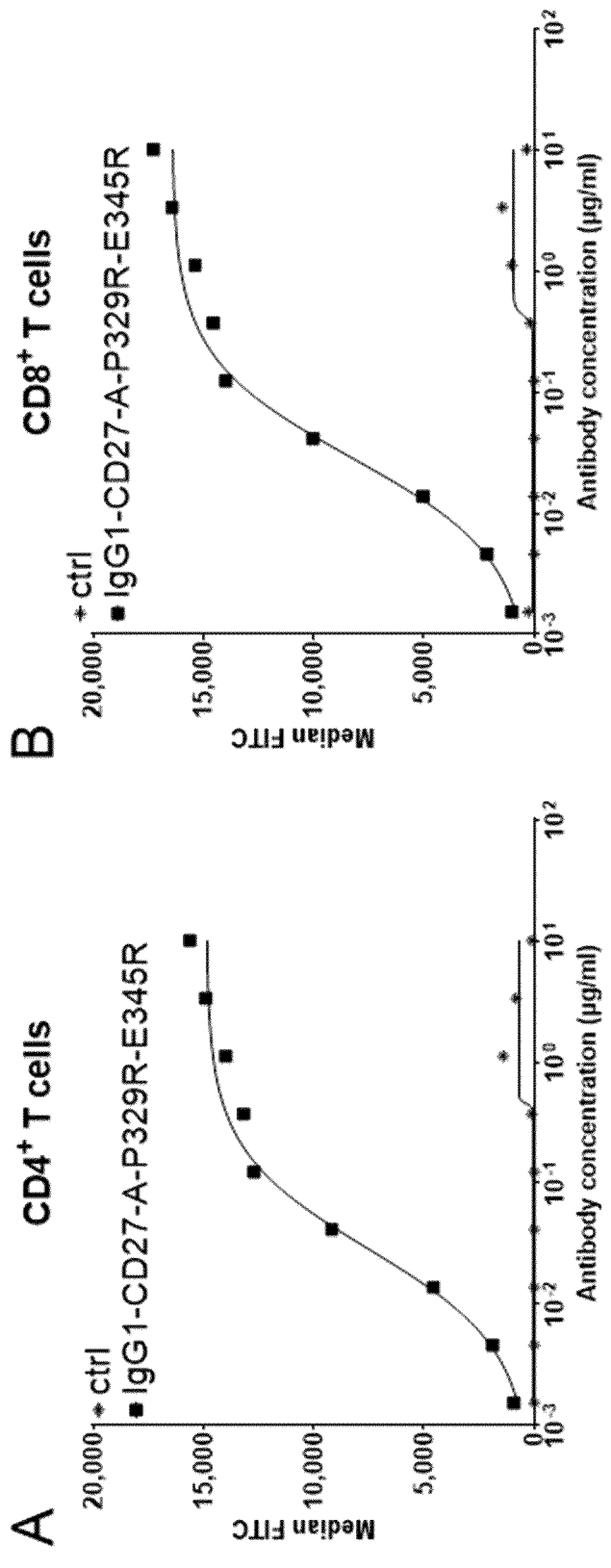

A
CD4+ T cells
ctrl
IgG1-CD27-A-P329R-E345R
Median FITC
20,000
15,000
10,000
5,000
0
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/ml)
B
CD8+ T cells
ctrl
IgG1-CD27-A-P329R-E345R
Median FITC
20,000
15,000
10,000
5,000
0
10-3
10-2
10-1
100
101
102
Antibody concentration (µg/ml)

**Figure 9**

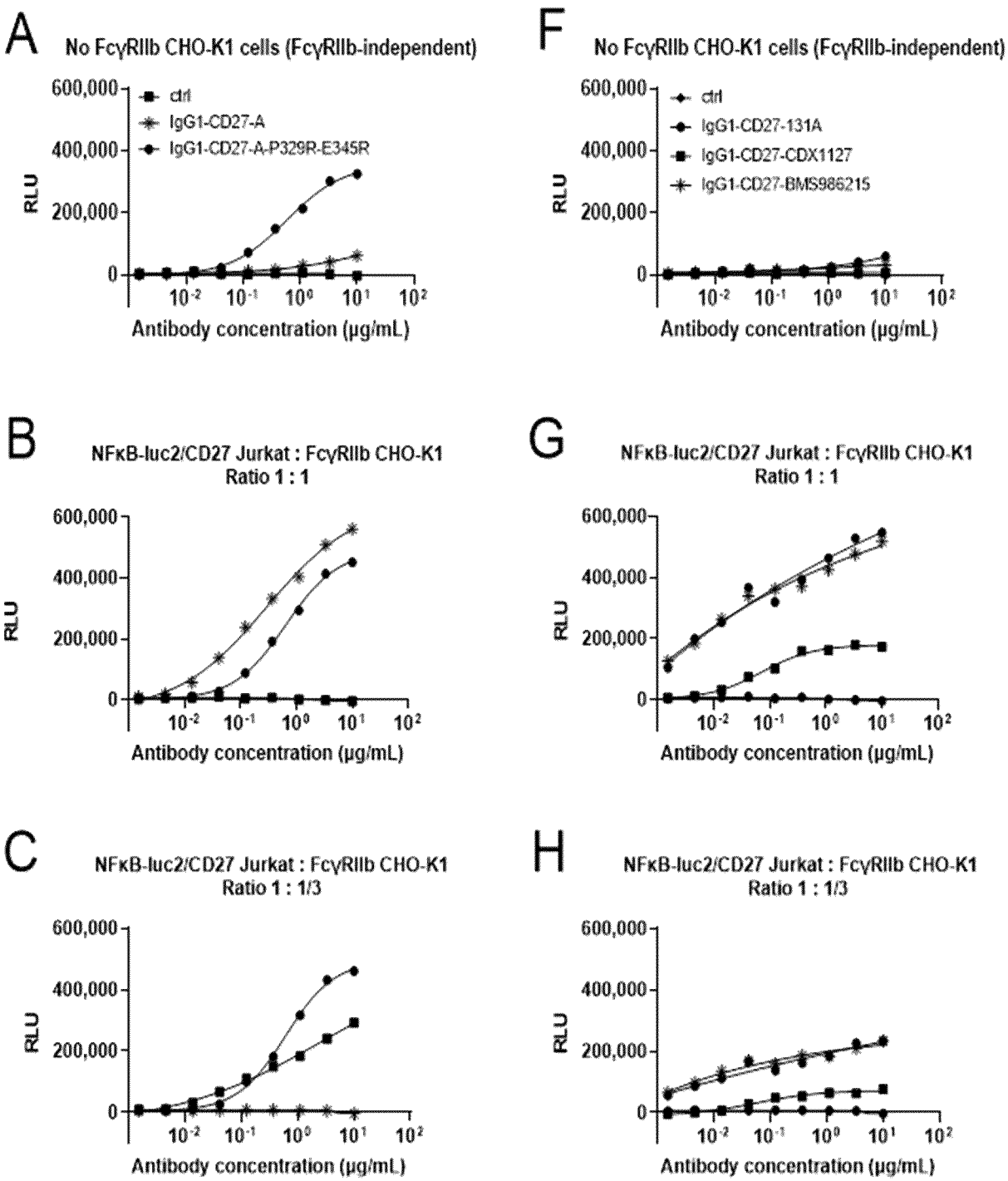

A
No FcγRIIb CHO-K1 cells (FcγRIIb-independent)
ctrl
IgG1-CD27-A
IgG1-CD27-A-P329R-E345R
RLU
Antibody concentration (µg/mL)
F
No FcγRIIb CHO-K1 cells (FcγRIIb-independent)
ctrl
IgG1-CD27-131A
IgG1-CD27-CDX1127
IgG1-CD27-BMS986215
RLU
Antibody concentration (µg/mL)
B
NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1
Ratio 1 : 1
RLU
Antibody concentration (µg/mL)
G
NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1
Ratio 1 : 1
RLU
Antibody concentration (µg/mL)
C
NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1
Ratio 1 : 1/3
RLU
Antibody concentration (µg/mL)
H
NFκB-luc2/CD27 Jurkat : FcγRIIb CHO-K1
Ratio 1 : 1/3
RLU
Antibody concentration (µg/mL)

**Figure 9 - continued**

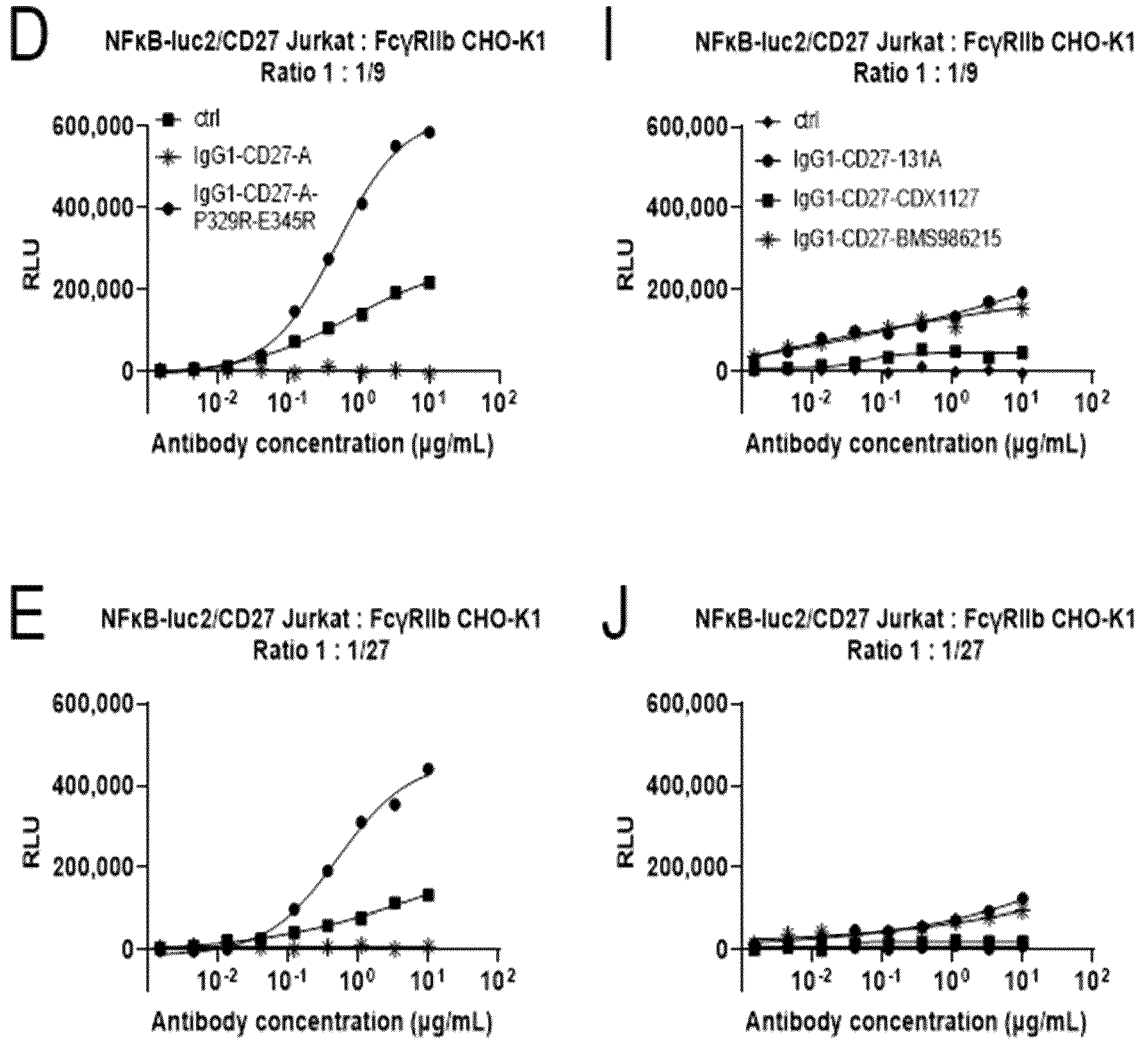


**Figure 10**

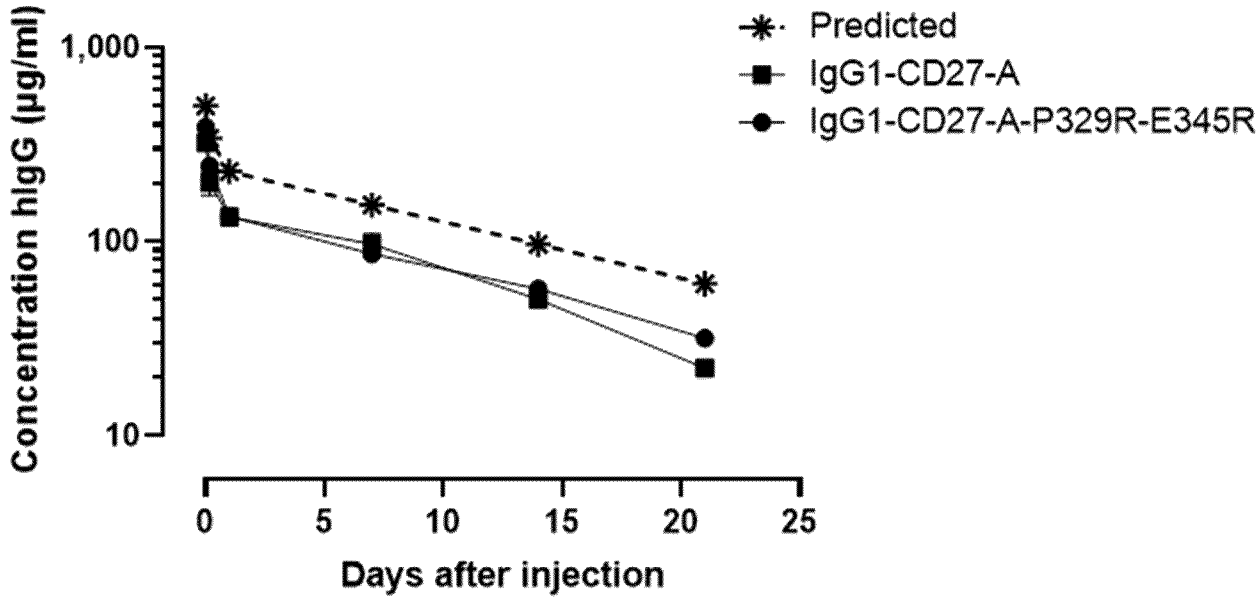

Predicted
IgG1-CD27-A
IgG1-CD27-A-P329R-E345R
Concentration hIgG (µg/ml)
1,000
100
10
0
5
10
15
20
25
Days after injection

**Figure 11**

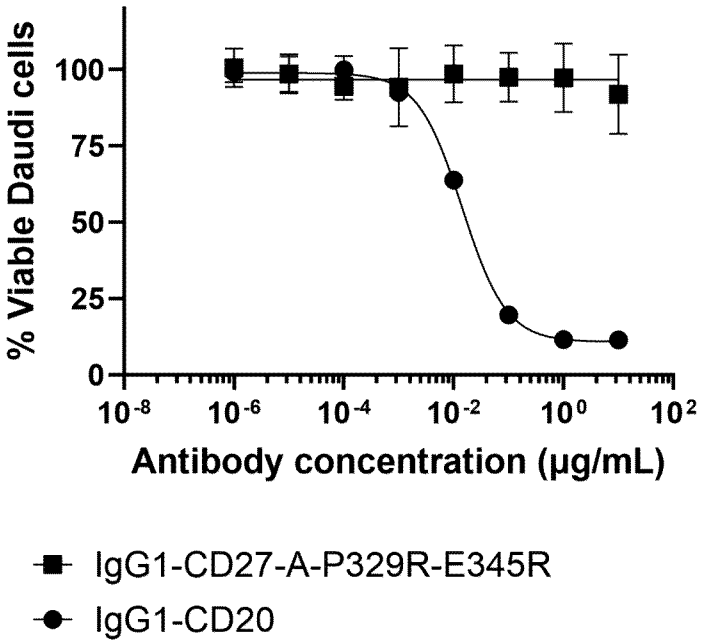



**Figure 12**

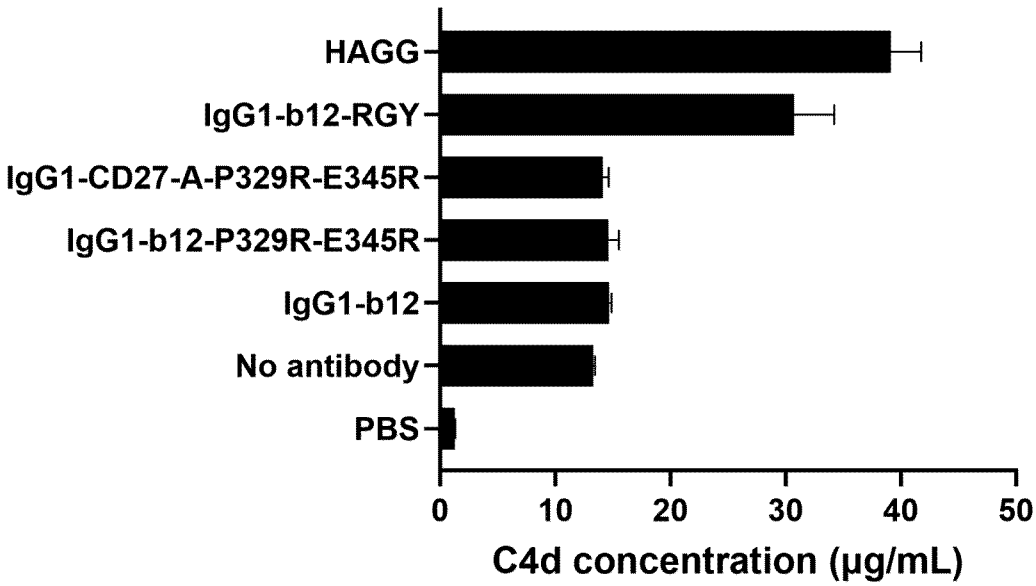


**Figure 13**

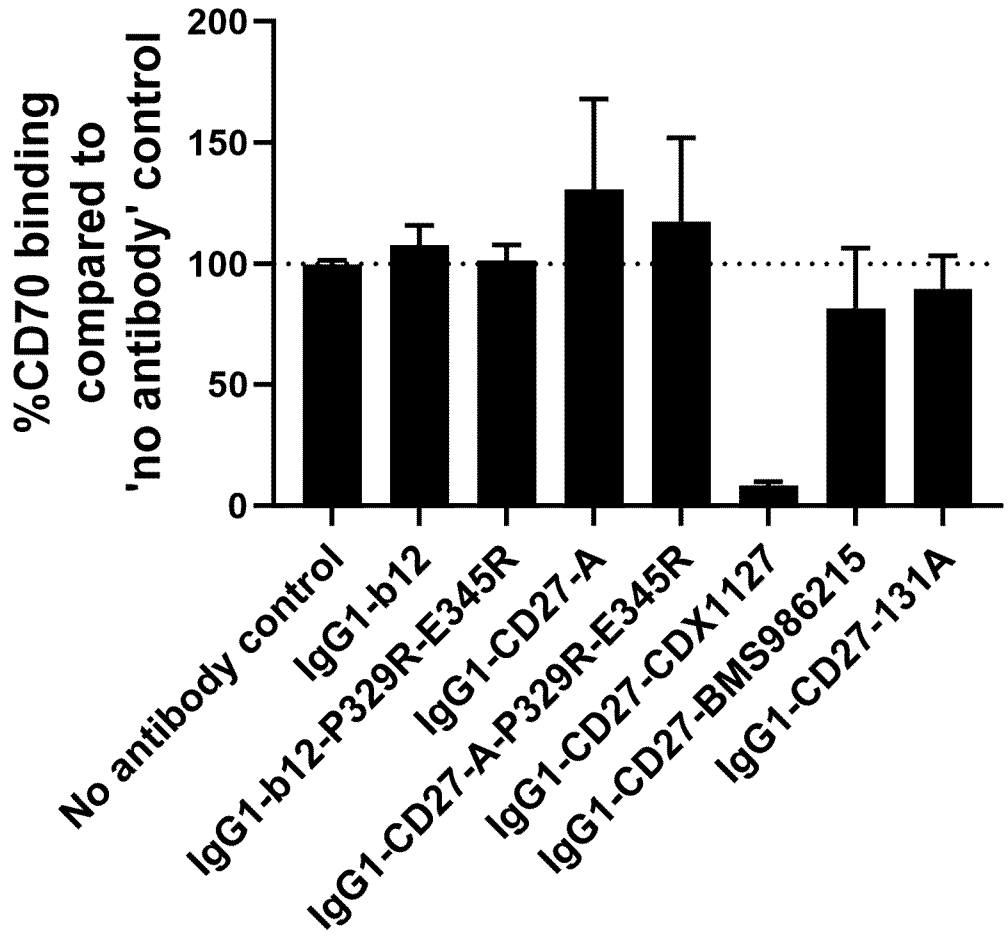
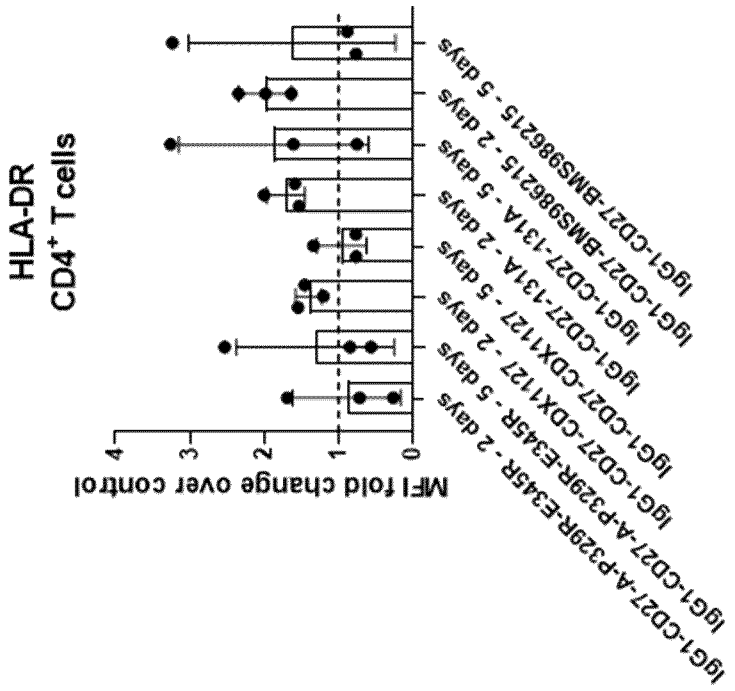
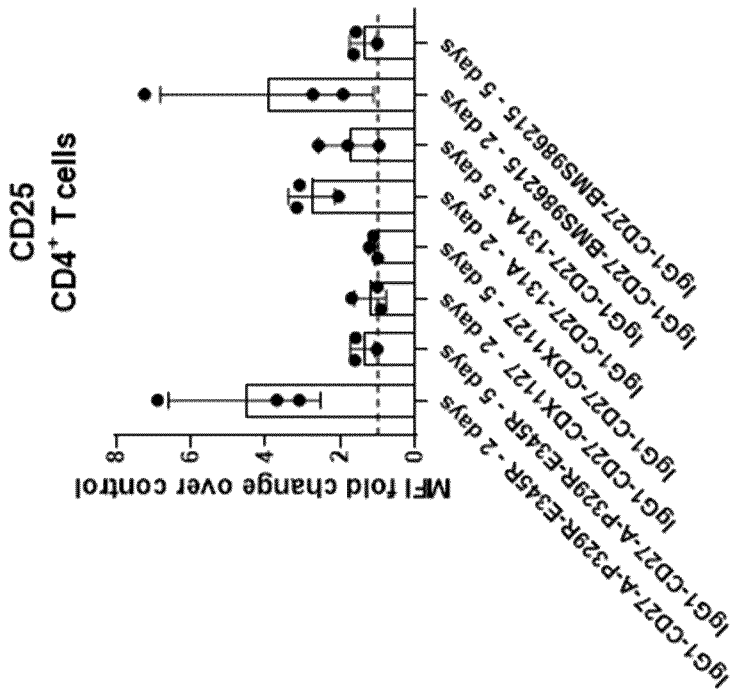
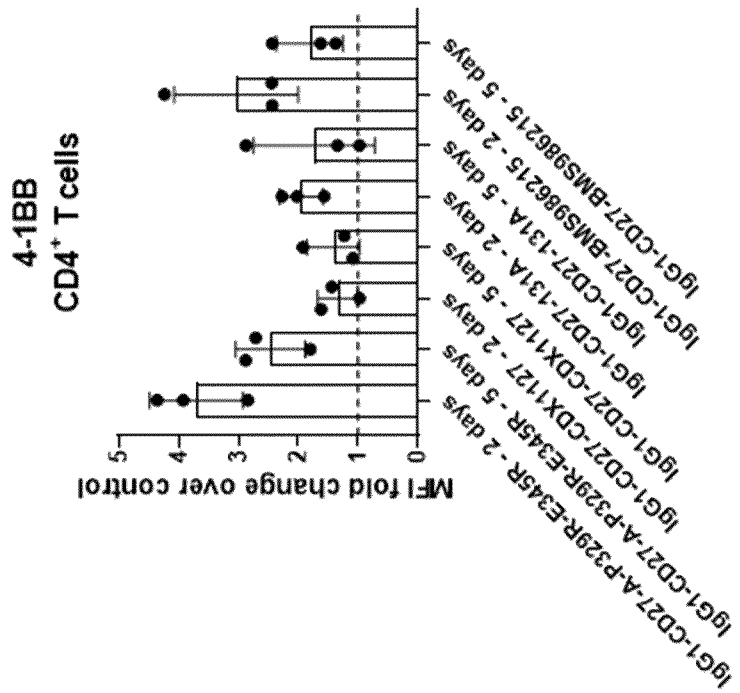

%CD70 binding compared to 'no antibody' control
200
150
100
50
0
No antibody control
IgG1-b12
IgG1-b12-P329R-E345R
IgG1-CD27-A
IgG1-CD27-A-P329R-E345R
IgG1-CD27-CDX1127
IgG1-CD27-BMS986215
IgG1-CD27-131A

Figure 14

A


HLA-DR
CD4+ T cells
MFI fold change over control
0
1
2
3
4
IgG1-CD27-A-P329R-E345R - 2 days
IgG1-CD27-A-P329R-E345R - 5 days
IgG1-CD27-CDX1127 - 2 days
IgG1-CD27-CDX1127 - 5 days
IgG1-CD27-131A - 2 days
IgG1-CD27-131A - 5 days
IgG1-CD27-BMS986215 - 2 days
IgG1-CD27-BMS986215 - 5 days


CD25
CD4+ T cells
MFI fold change over control
0
2
4
6
8
IgG1-CD27-A-P329R-E345R - 2 days
IgG1-CD27-A-P329R-E345R - 5 days
IgG1-CD27-CDX1127 - 2 days
IgG1-CD27-CDX1127 - 5 days
IgG1-CD27-131A - 2 days
IgG1-CD27-131A - 5 days
IgG1-CD27-BMS986215 - 2 days
IgG1-CD27-BMS986215 - 5 days



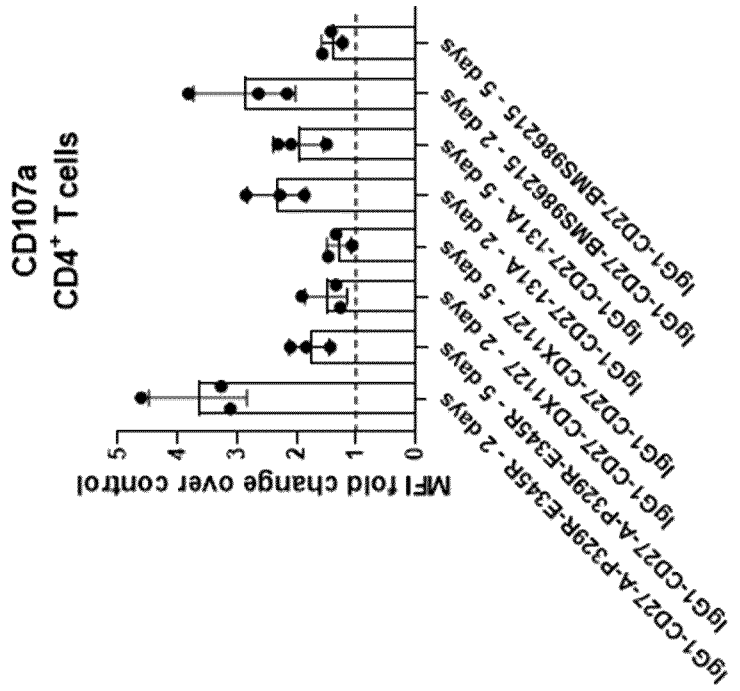
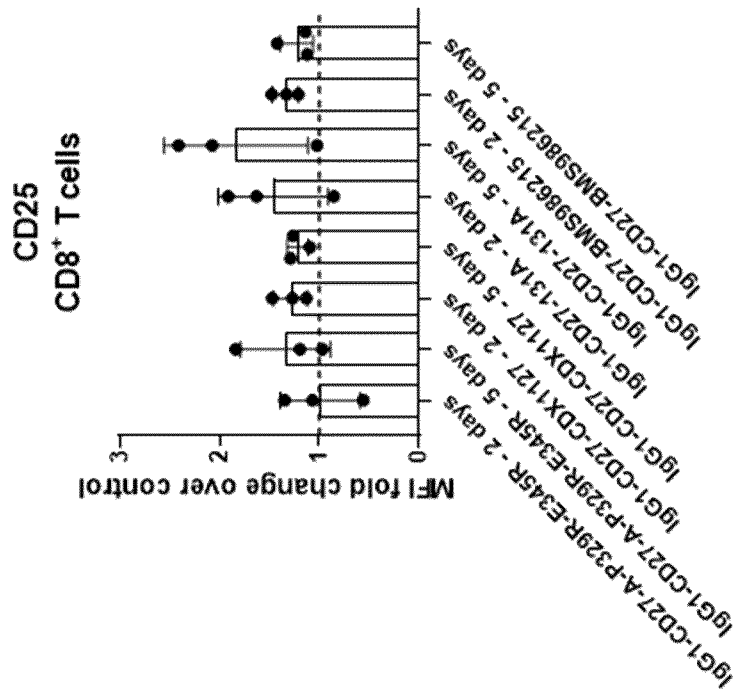




**Figure 14A** – *continued*


CD25
CD8+ T cells
MFI fold change over control
0
1
2
3
IgG1-CD27-A-P329R-E345R - 2 days
IgG1-CD27-A-P329R-E345R - 5 days
IgG1-CD27-CDX1127 - 2 days
IgG1-CD27-CDX1127 - 5 days
IgG1-CD27-131A - 2 days
IgG1-CD27-131A - 5 days
IgG1-CD27-BMS986215 - 2 days
IgG1-CD27-BMS986215 - 5 days

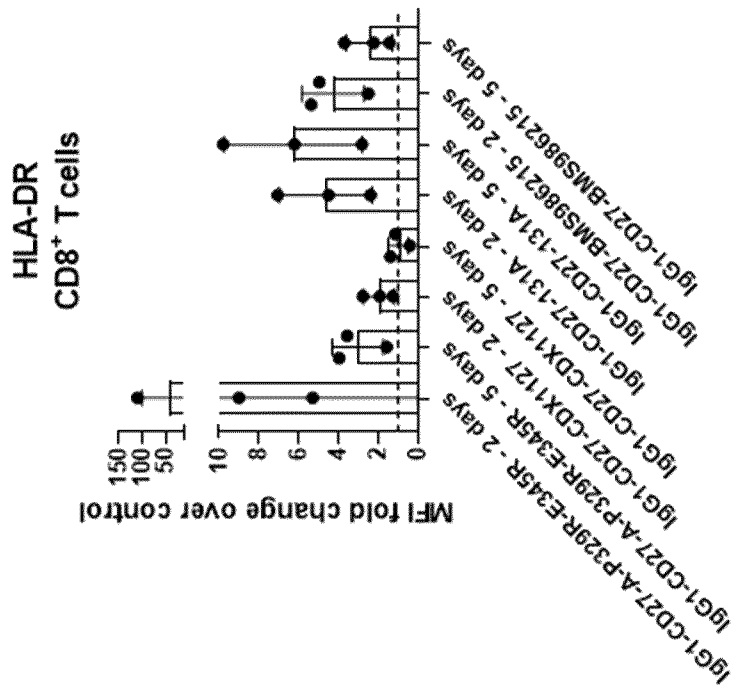
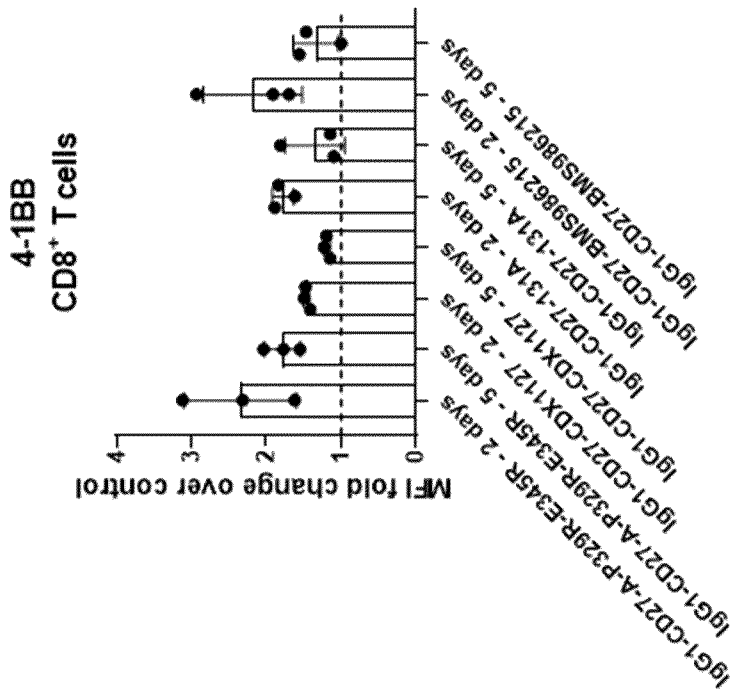

HLA-DR
CD8+ T cells
MFI fold change over control
0
2
4
6
8
10
50
100
150
IgG1-CD27-A-P329R-E345R - 2 days
IgG1-CD27-A-P329R-E345R - 5 days
IgG1-CD27-CDX1127 - 2 days
IgG1-CD27-CDX1127 - 5 days
IgG1-CD27-131A - 2 days
IgG1-CD27-131A - 5 days
IgG1-CD27-BMS986215 - 2 days
IgG1-CD27-BMS986215 - 5 days

Figure 14B



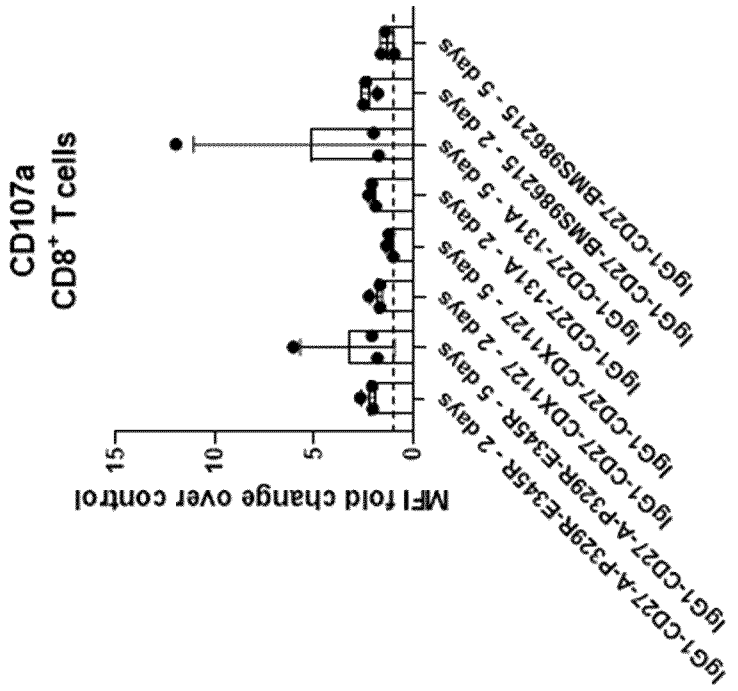




**Figure 14B** – *continued*

**Figure 15**

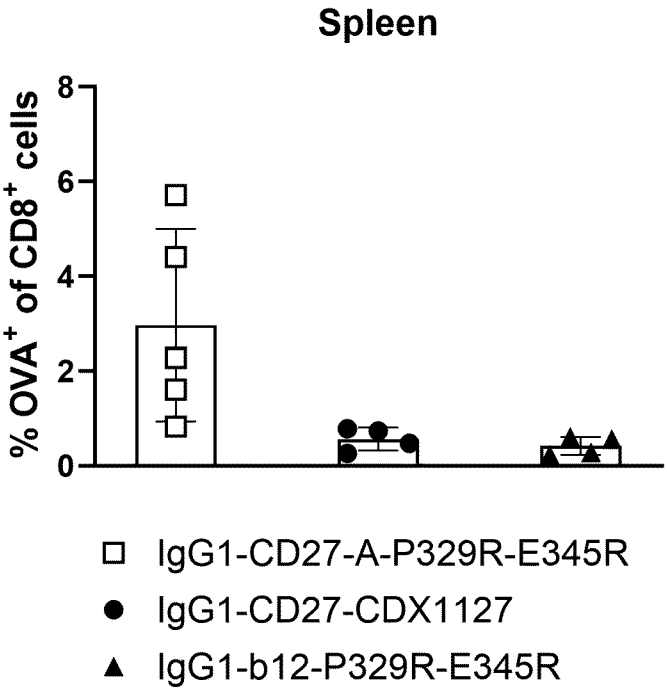



**Figure 16**

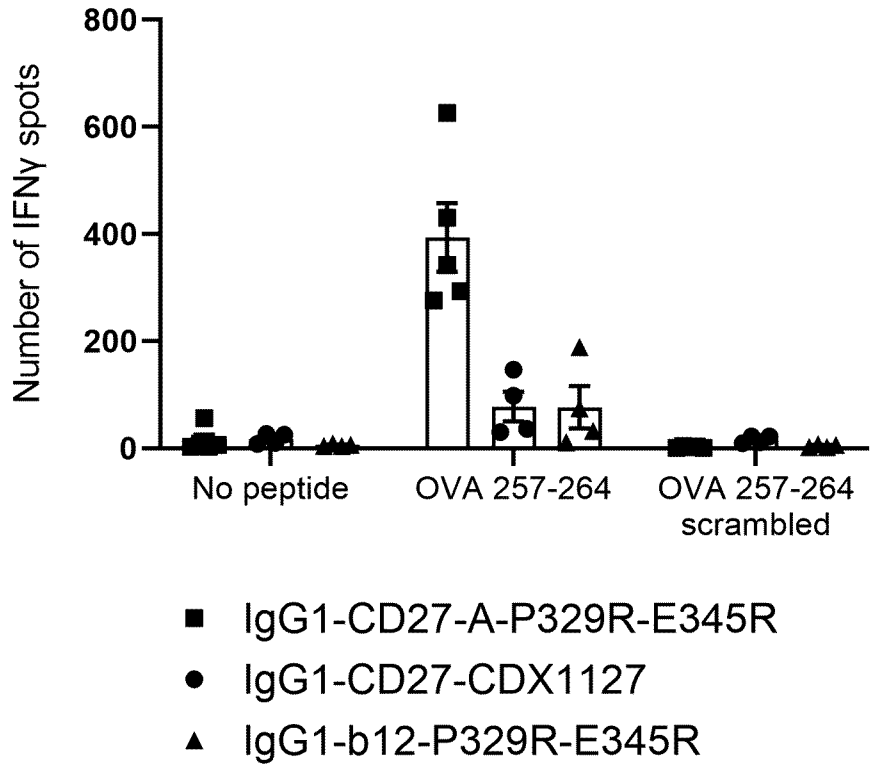


**Figure 17**

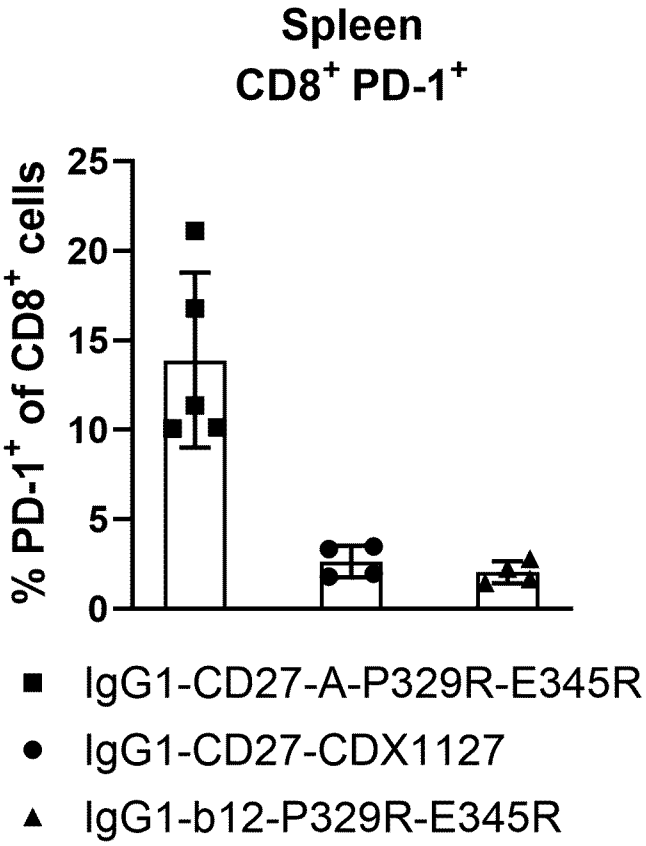

Spleen
CD8+ PD-1+
% PD-1+ of CD8+ cells
25
20
15
10
5
0
IgG1-CD27-A-P329R-E345R
IgG1-CD27-CDX1127
IgG1-b12-P329R-E345R

Figure 18

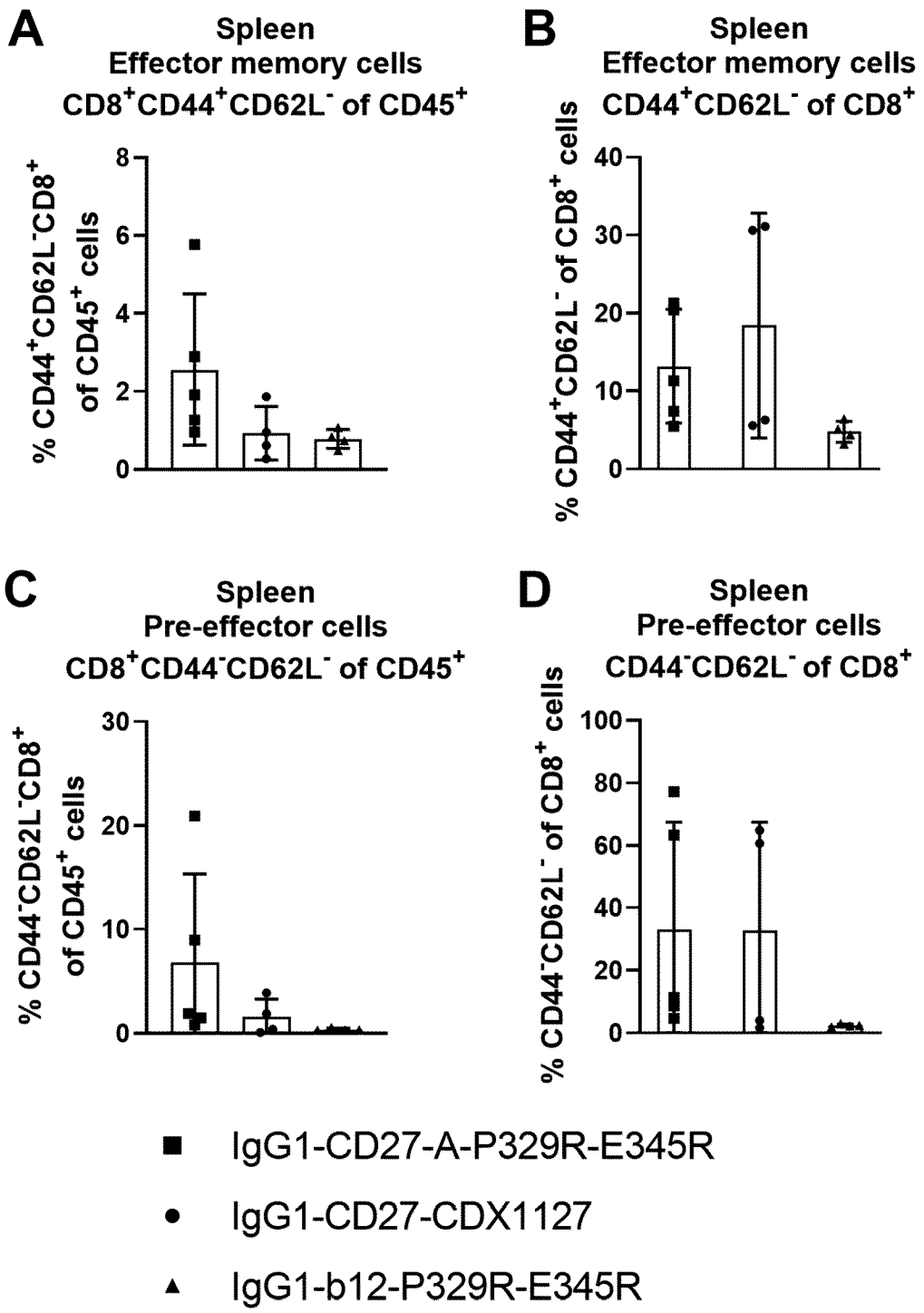

A
Spleen
Effector memory cells
CD8+CD44+CD62L- of CD45+
% CD44+CD62L-CD8+ of CD45+ cells
0
2
4
6
8
B
Spleen
Effector memory cells
CD44+CD62L- of CD8+
% CD44+CD62L- of CD8+ cells
0
10
20
30
40
C
Spleen
Pre-effector cells
CD8+CD44-CD62L- of CD45+
% CD44-CD62L-CD8+ of CD45+ cells
0
10
20
30
D
Spleen
Pre-effector cells
CD44-CD62L- of CD8+
% CD44-CD62L- of CD8+ cells
0
20
40
60
80
100
IgG1-CD27-A-P329R-E345R
IgG1-CD27-CDX1127
IgG1-b12-P329R-E345R

**Figure 19**

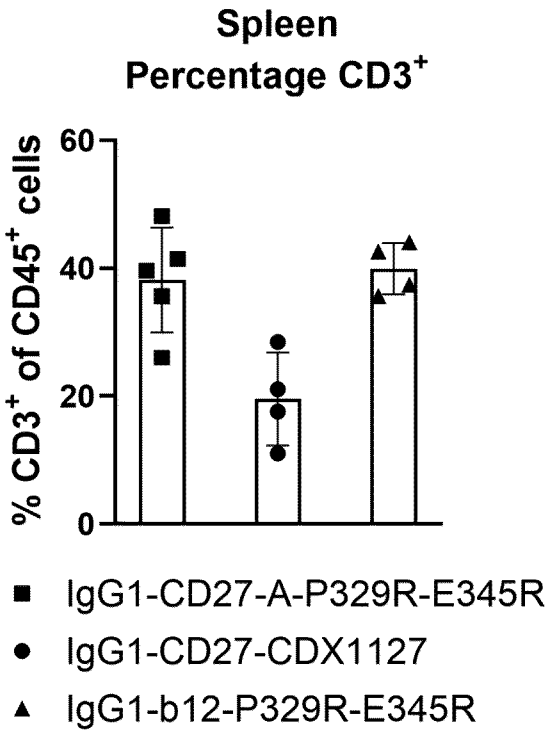

Spleen
Percentage CD3+
% CD3+ of CD45+ cells
60
40
20
0
IgG1-CD27-A-P329R-E345R
IgG1-CD27-CDX1127
IgG1-b12-P329R-E345R

Figure 20

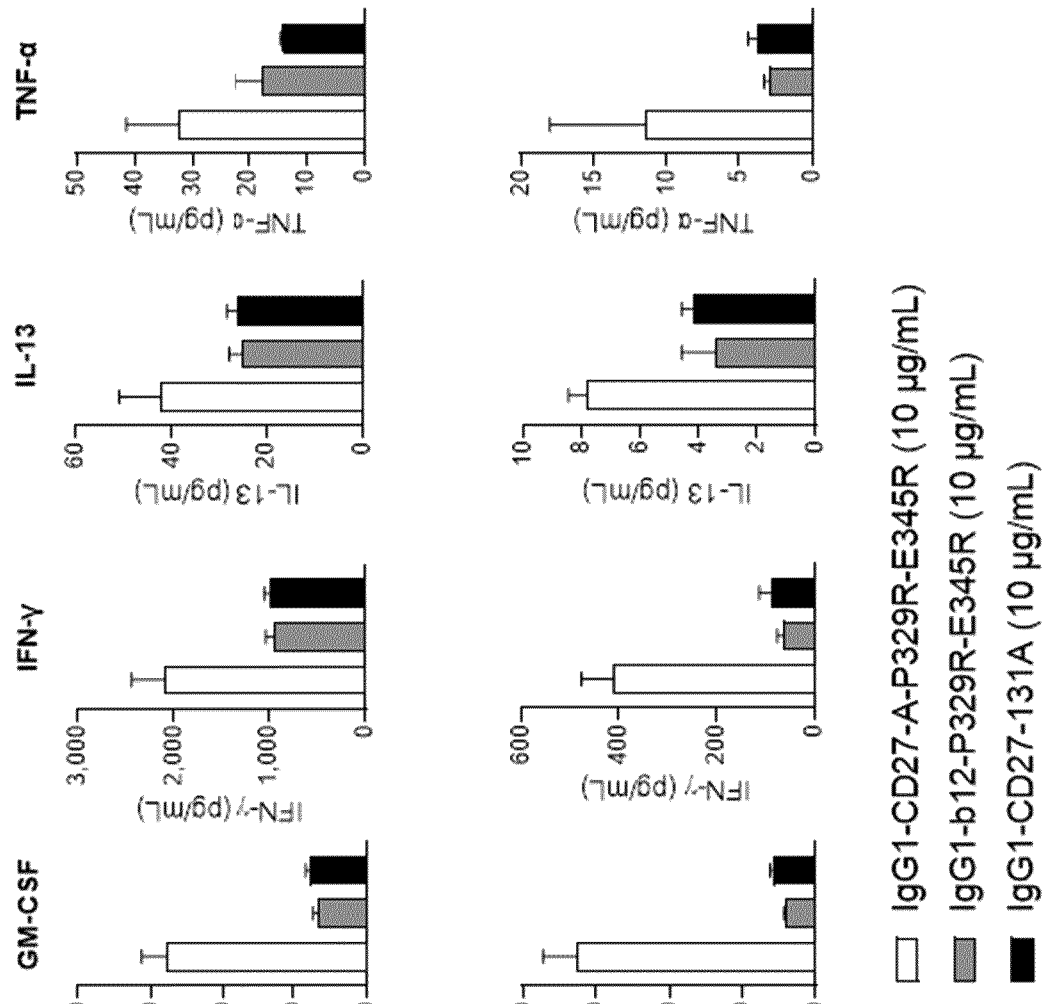

A
Endogenous PD1
GM-CSF
GM-CSF (pg/mL)
IFN-γ
IFN-γ (pg/mL)
IL-13
IL-13 (pg/mL)
TNF-α
TNF-α (pg/mL)
B
PD1 overexpression
GM-CSF (pg/mL)
IFN-γ (pg/mL)
IL-13 (pg/mL)
TNF-α (pg/mL)
IgG1-CD27-A-P329R-E345R (10 µg/mL)
IgG1-b12-P329R-E345R (10 µg/mL)
IgG1-CD27-131A (10 µg/mL)

Figure 21

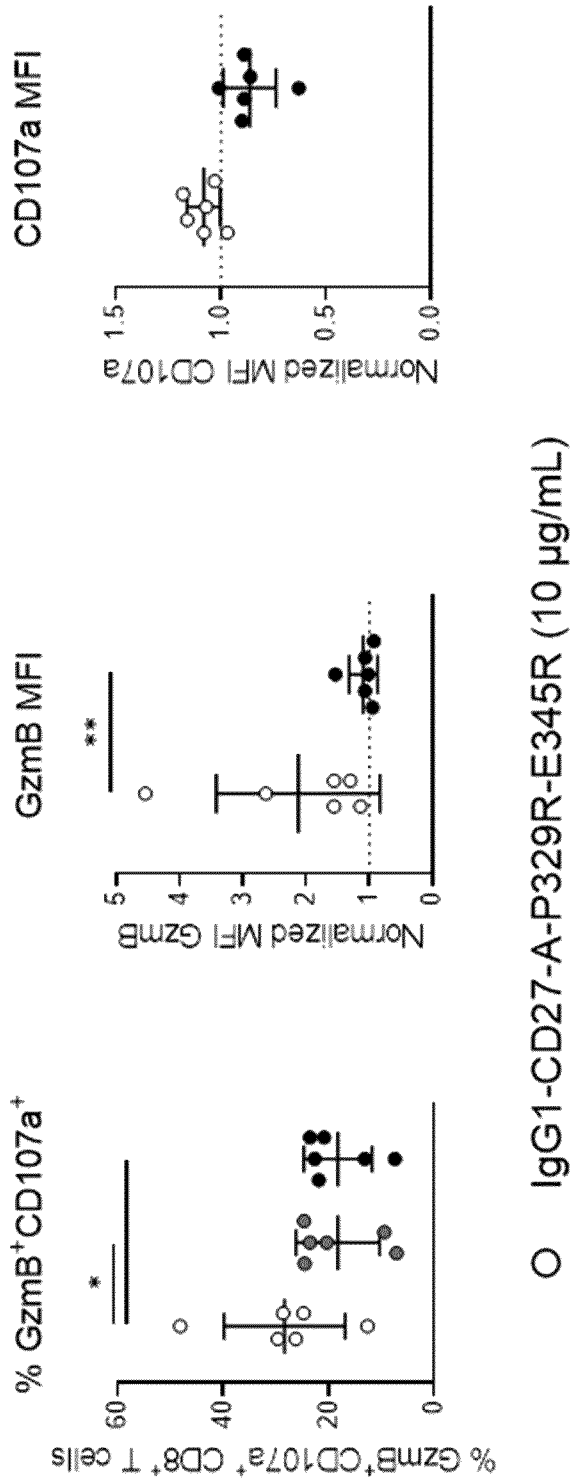

% GzmB+CD107a+
% GzmB+CD107a+ CD8+ T cells
60
40
20
0
*
GzmB MFI
Normalized MFI GzmB
5
4
3
2
1
0
**
CD107a MFI
Normalized MFI CD107a
1.5
1.0
0.5
0.0
IgG1-CD27-A-P329R-E345R (10 µg/mL)
IgG1-b12-P329R-E345R (10 µg/mL)
IgG1-CD27-131A (10 µg/mL)

**Figure 22**

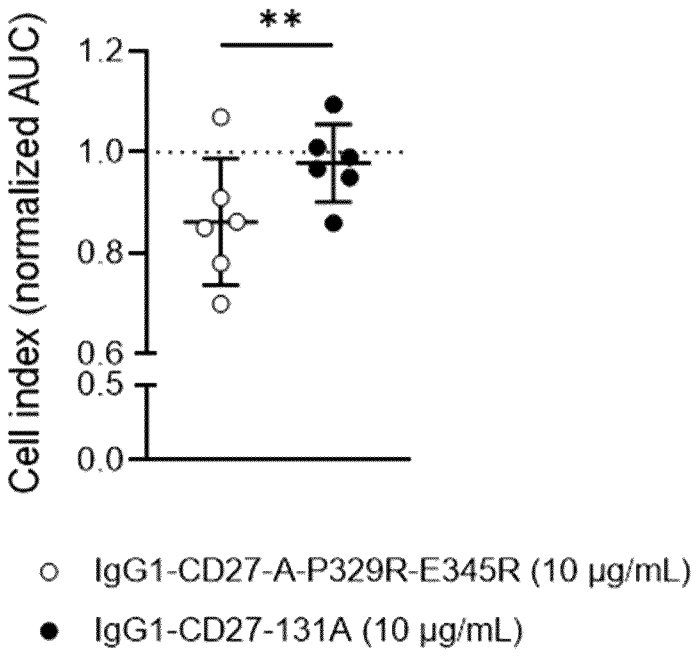



**Figure 23**

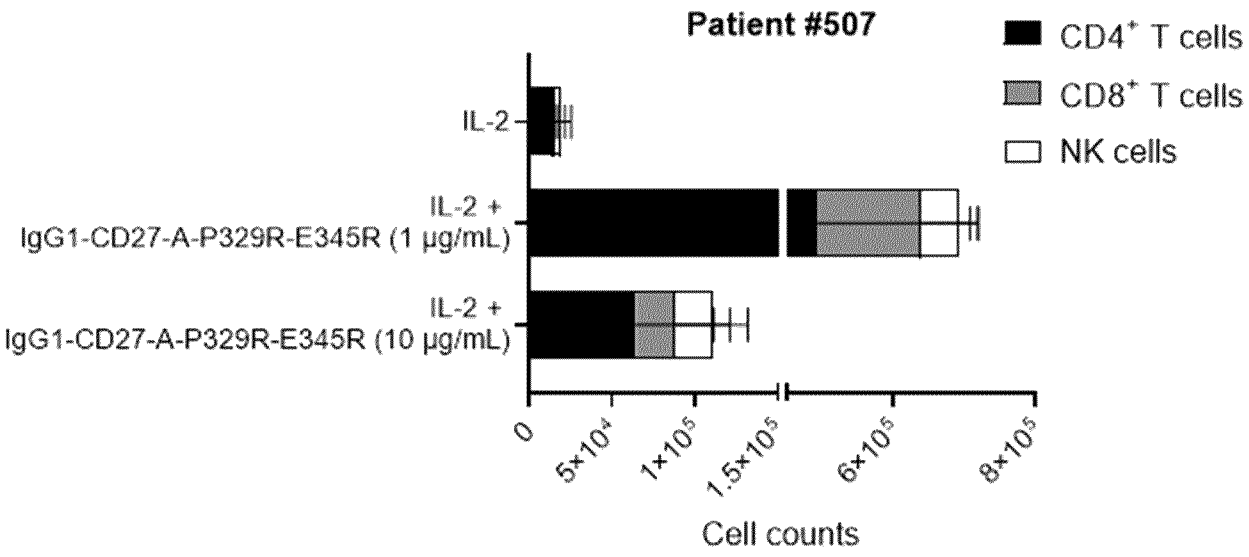


**Figure 24**

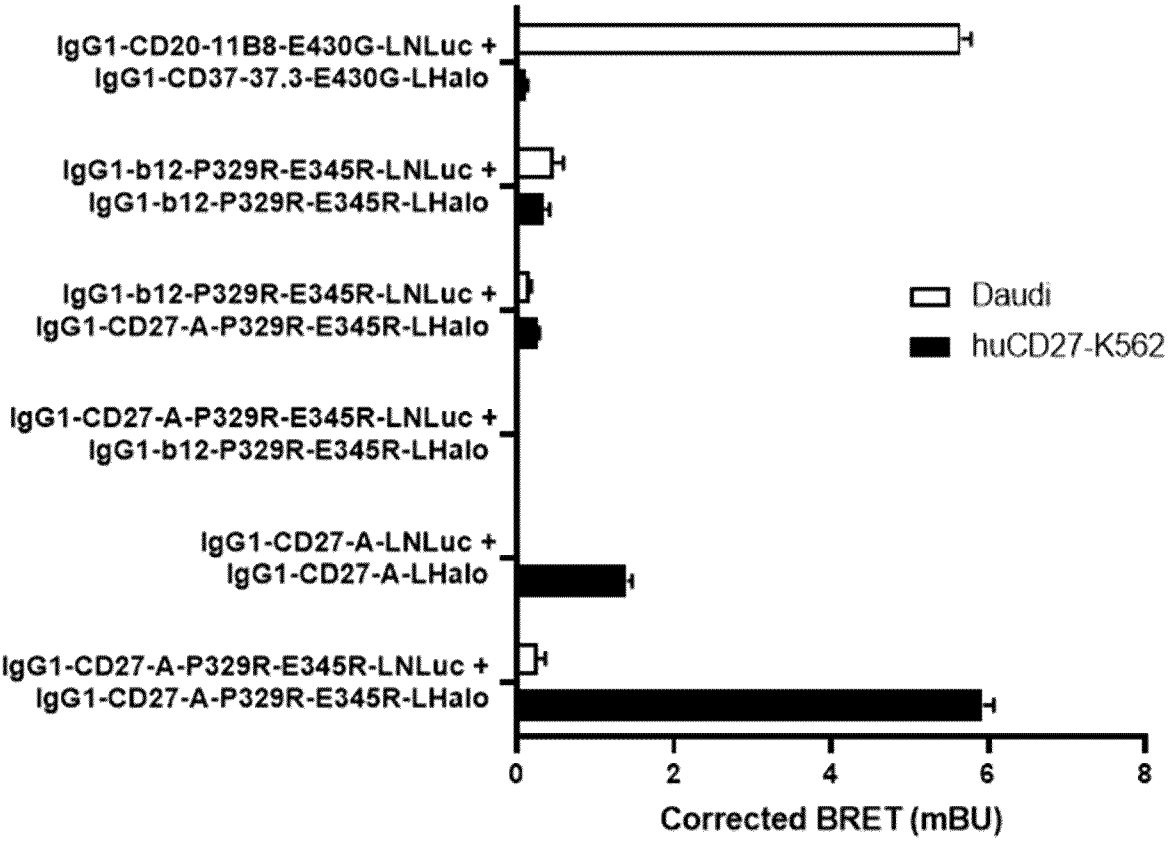

IgG1-CD20-11B8-E430G-LNLuc +
IgG1-CD37-37.3-E430G-LHalo
IgG1-b12-P329R-E345R-LNLuc +
IgG1-b12-P329R-E345R-LHalo
IgG1-b12-P329R-E345R-LNLuc +
IgG1-CD27-A-P329R-E345R-LHalo
IgG1-CD27-A-P329R-E345R-LNLuc +
IgG1-b12-P329R-E345R-LHalo
IgG1-CD27-A-LNLuc +
IgG1-CD27-A-LHalo
IgG1-CD27-A-P329R-E345R-LNLuc +
IgG1-CD27-A-P329R-E345R-LHalo
Daudi
huCD27-K562
0
2
4
6
8
Corrected BRET (mBU)

**Figure 25**

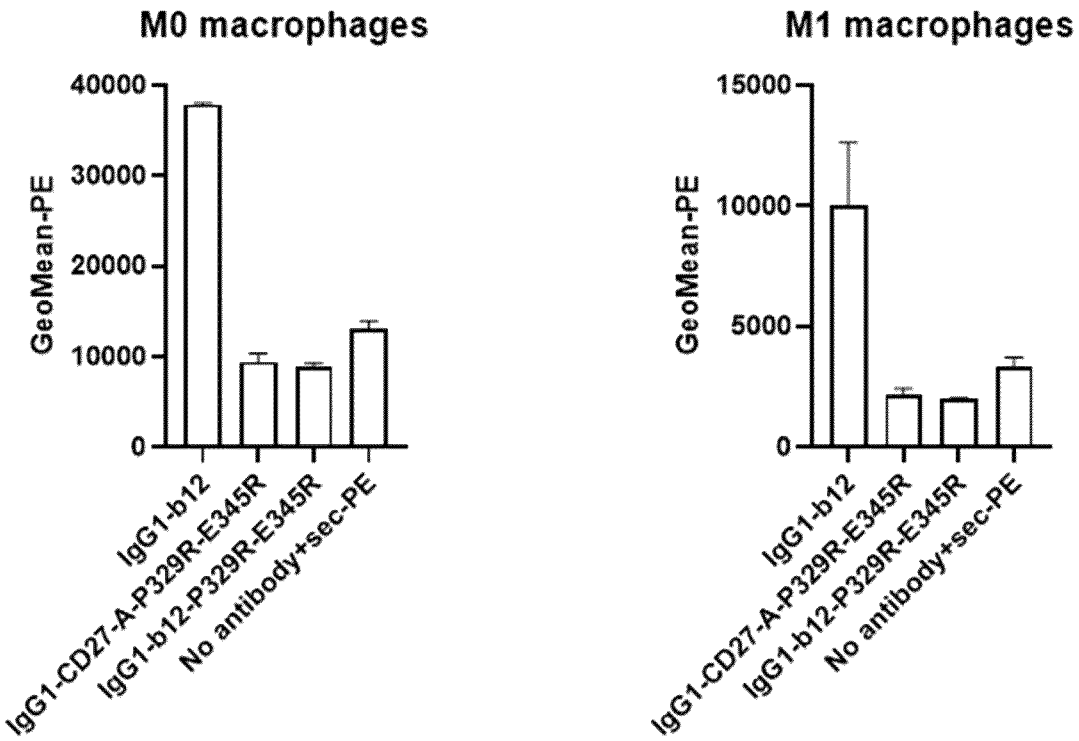

M0 macrophages
GeoMean-PE
40000
30000
20000
10000
0
IgG1-b12
IgG1-CD27-A-P329R-E345R
IgG1-b12-P329R-E345R
No antibody+sec-PE
M1 macrophages
GeoMean-PE
15000
10000
5000
0
IgG1-b12
IgG1-CD27-A-P329R-E345R
IgG1-b12-P329R-E345R
No antibody+sec-PE

**Figure 26**

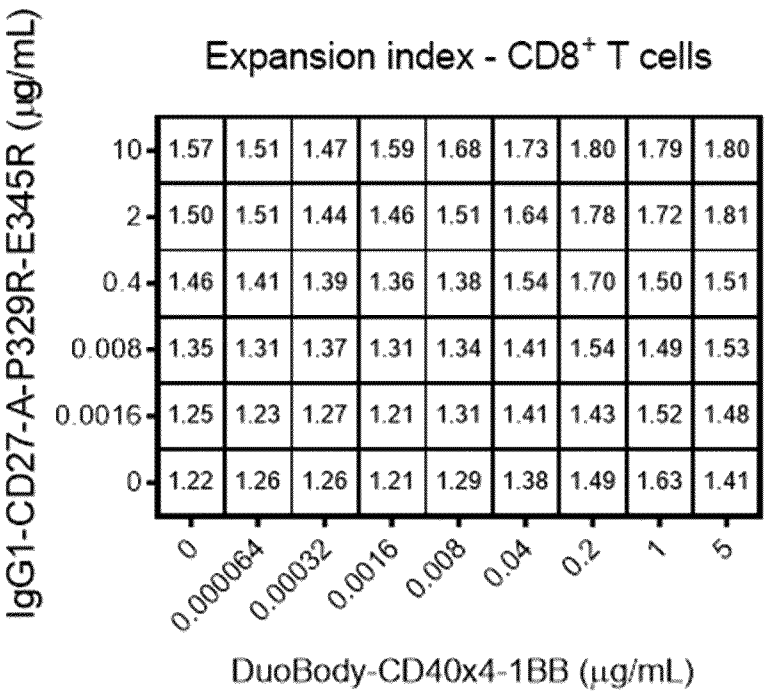

Expansion index - CD8+ T cells
IgG1-CD27-A-P329R-E345R (µg/mL)
10 1.57 1.51 1.47 1.59 1.68 1.73 1.80 1.79 1.80
2 1.50 1.51 1.44 1.46 1.51 1.64 1.78 1.72 1.81
0.4 1.46 1.41 1.39 1.36 1.38 1.54 1.70 1.50 1.51
0.008 1.35 1.31 1.37 1.31 1.34 1.41 1.54 1.49 1.53
0.0016 1.25 1.23 1.27 1.21 1.31 1.41 1.43 1.52 1.48
0 1.22 1.26 1.26 1.21 1.29 1.38 1.49 1.63 1.41
0
0.00064
0.00032
0.0016
0.008
0.04
0.2
1
5
DuoBody-CD40x4-1BB (µg/mL)

Figure 27

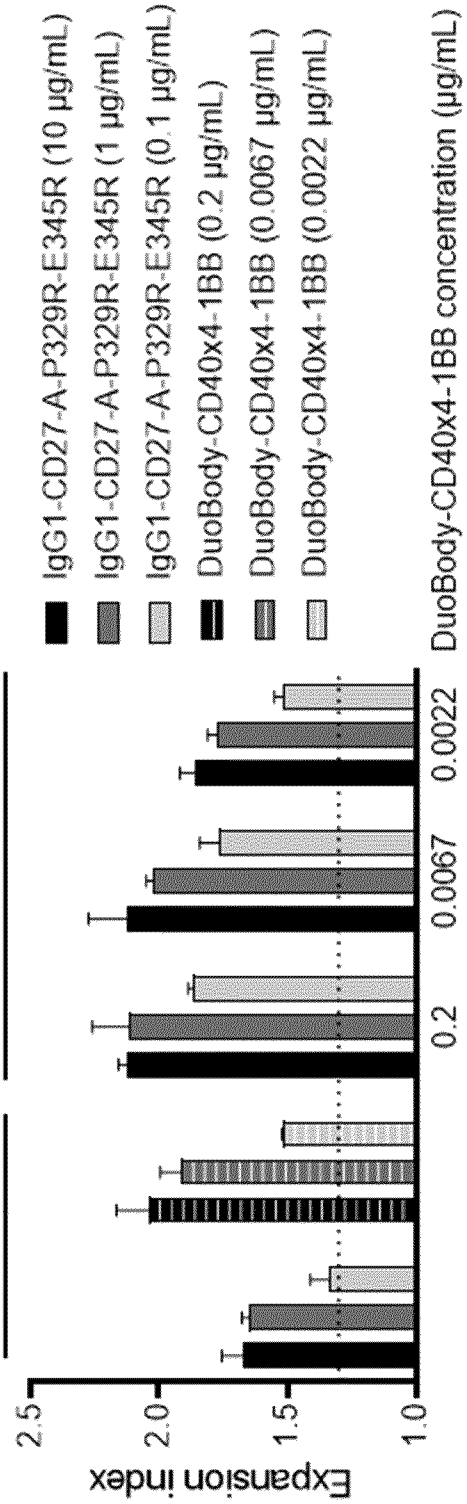
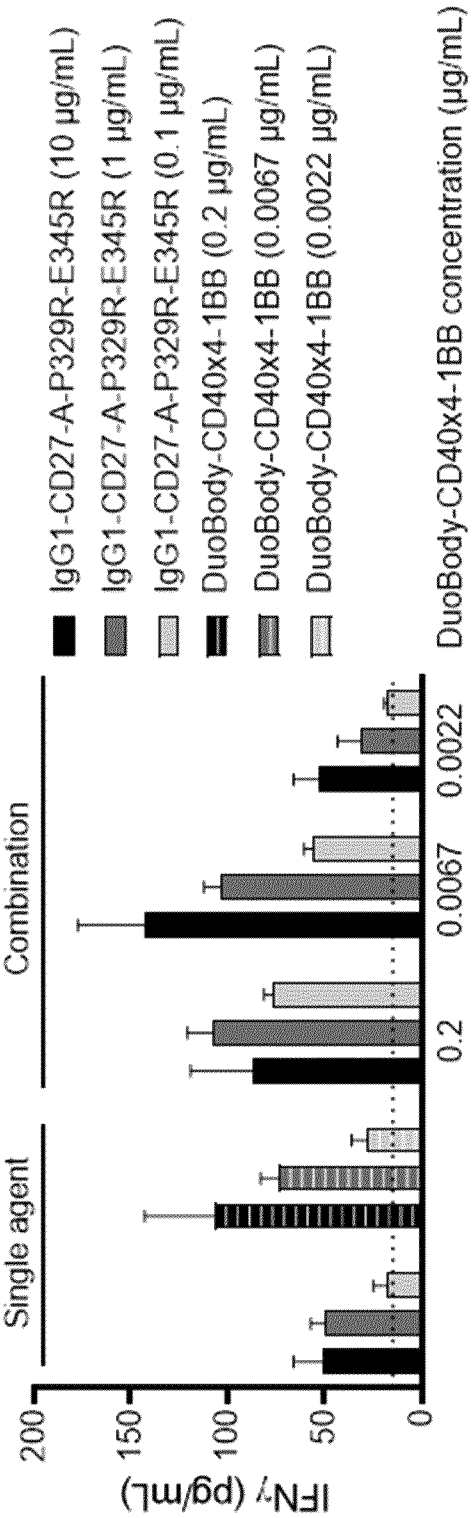

Single agent
Combination
IgG1-CD27-A-P329R-E345R (10 µg/mL)
IgG1-CD27-A-P329R-E345R (1 µg/mL)
IgG1-CD27-A-P329R-E345R (0.1 µg/mL)
DuoBody-CD40x4-1BB (0.2 µg/mL)
DuoBody-CD40x4-1BB (0.0067 µg/mL)
DuoBody-CD40x4-1BB (0.0022 µg/mL)
Expansion index
2.5
2.0
1.5
1.0
0.2
0.0067
0.0022
DuoBody-CD40x4-1BB concentration (µg/mL)

Figure 28


IFNγ
Single agent
Combination
IFNγ (pg/mL)
200
150
100
50
0
0.2
0.0067
0.0022
DuoBody-CD40x4-1BB concentration (µg/mL)
IgG1-CD27-A-P329R-E345R (10 µg/mL)
IgG1-CD27-A-P329R-E345R (1 µg/mL)
IgG1-CD27-A-P329R-E345R (0.1 µg/mL)
DuoBody-CD40x4-1BB (0.2 µg/mL)
DuoBody-CD40x4-1BB (0.0067 µg/mL)
DuoBody-CD40x4-1BB (0.0022 µg/mL)

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/062798

**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:

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/062798

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P35/00 C07K16/28
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)
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, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | TOMOYASU UNO ET AL: "Eradication of established tumors in mice by a combination antibody-based therapy", NATURE MEDICINE, vol. 12, no. 6, 7 May 2006 (2006-05-07), pages 693-698, XP055123624, ISSN: 1078-8956, DOI: 10.1038/nm1405 abstract<br>-----<br>WO 2018/011421 A1 (GENMAB AS [DK]; BIONTECH AG [DE])<br>18 January 2018 (2018-01-18)<br>page 1, line 13 - page 2, line 10<br>page 117, line 34 - page 118, line 16<br>-----<br>-/-- | 1-97<br><br>1-97 |

[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 |
|---|---|
| 12 July 2023 | 19/07/2023 |

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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International application No
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