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(54) **Title:** MONOVALENT CD27 ANTIBODIES

(57) **Abstract:** The present invention relates to a monovalent antibody, such as a Fab fragment, which is capable of specifically binding CD27 and reducing CD27 induced signalling. The present invention also relates to a fusion protein or conjugate comprising the monovalent antibody of the invention and a half-life extending moiety. The monovalent antibody, fusion protein or conjugate of the invention may be suitable for use as a medicament, such as in the treatment of autoimmune diseases in which suppression of CD27 signaling is desirable.



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MONOVALENT CD27 ANTIBODIES

TECHNICAL FIELD

The invention relates to monovalent antibodies that are capable of binding CD27 and that antagonise its function, to pharmaceutical formulations comprising CD27 monovalent antibodies and to their uses as medicaments.

BACKGROUND

CD27 (TNFRSF7), a member of the TNF-receptor superfamily, is a lymphocyte co-stimulatory molecule that regulates T-cell, natural killer (NK) cell, B-cell, and plasma cell function, survival, and differentiation. TNFR family members are characterised by repetitive patterns of several cysteine-rich domains (CRDs). A pre-ligand-binding assembly domain (PLAD domain), located in CRD1, is common among the TNFR superfamily members and promotes ligand-independent multimer formation. CD27 is required for the generation and long-term maintenance of T-cell immunity. It binds to ligand CD70, and plays a key role in regulating B-cell activation and immunoglobulin synthesis. CD70 binding to CD27 induces receptor phosphorylation, calcium fluxes and signaling via adaptor molecules of the TRAF-family coupling to the NF- κ B and SAPK/JNK pathways. Adaptor proteins TRAF2 and TRAF5 have been shown to mediate the signaling process of CD27. Immunologically, lack of CD27 expression has been associated with impaired T cell-dependent B-cell responses and T-cell dysfunction (J Allergy Clin Immunol, 2012: 129, 3).

Monoclonal (bivalent) antibodies that specifically bind CD27 are known in the art and are both commercially available and disclosed in publications such as WO11130434. Such known antibodies have agonistic properties, caused by their ability to cross-link CD27 receptors. Antibody crosslinking can induce receptor signaling in the absence of ligand CD70 or increase receptor signaling in the presence of CD70.

There is still a need in the art for a CD27 molecule, such as an antibody, that is capable of antagonising CD27 function and which may be used as a medicament where suppression of T-cell function or T cell-dependent B-cell responses is desirable.

SUMMARY

The present invention relates to a monovalent antibody which is capable of specifically binding CD27 and reducing CD27 induced signalling. An antibody according to the invention may be capable of inhibiting B-cell IgG secretion by at least 50%. An antibody according to the invention may have an IC₅₀ value that is less than 10nM. An antibody

according to the invention may not be capable of competing with CD70 for binding to CD27. An antibody according to the invention may reduce CD27/CD70 mediated co-stimulation of CD4+ and/or CD8+ T cells by more than 50%.

5 The present invention also relates to fusion proteins or conjugates comprising the monovalent antibody of the invention, as well as a half-life extending moiety.

The monovalent antibody, fusion protein or conjugate of the invention may be suitable for use as a medicament, such as in the treatment of autoimmune diseases in which suppression of CD27 signaling is desirable.

10 BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 represents the amino acid sequence of wild-type human CD27.

SEQ ID NO: 2 represents the nucleic acid sequence of the pMskj1626 (5'-3') primer.

SEQ ID NO: 3 represents the nucleic acid sequence of the PSqld1241 (5'-3') primer.

SEQ ID NO: 4 represents the nucleic acid sequence of the pMskj1630 (5'-3') primer.

15 SEQ ID NO: 5 represents the nucleic acid sequence of the PSqld1243 (5'-3') primer.

SEQ ID NO: 6 represents the nucleic acid sequence of the pSqld_353 (5'-3') primer.

SEQ ID NO: 7 represents the nucleic acid sequence of the pSqld_554 (5'-3') primer.

SEQ ID NO: 8 represents the nucleic acid sequence of NFkappaB binding sites, the minimal interferon promoter and the coding region of the Firefly luciferase gene.

20 SEQ ID NO: 9 represents the nucleic acid sequence of two heat shock elements and the minimal c-fos promoter.

SEQ ID NO: 10 represents the nucleic acid sequence of the first overlapping forward primer used for PCR amplification of the coding cDNA of human CD70 extracellular domain.

25 SEQ ID NO: 11 represents the nucleic acid sequence of the second overlapping forward primer used for PCR amplification of the coding cDNA of human CD70 extracellular domain.

SEQ ID NO: 12 represents the nucleic acid sequence of reverse primer used for PCR amplification of the coding cDNA of human CD70 extracellular domain.

30 SEQ ID NO: 13 represents the nucleic acid sequence of the coding region of CD70 with a FLAG-tag, HIS-tag and TEV cleavage site including EcoRI and NheI restriction sites.

SEQ ID NO: 14 represents the nucleic acid sequence encoding the human CD33 signal peptide.

SEQ ID NO: 15 represents the amino acid sequence of the human CD33 signal peptide.

SEQ ID NO: 16 represents the nucleic acid sequence of forward primer used for PCR amplifying the human CD27 coding cDNA.

SEQ ID NO: 17 represents the nucleic acid sequence of reverse primer used for PCR amplifying the human CD27 coding cDNA.

5 SEQ ID NO: 18 represents the sequence of forward primer used for PCR amplifying the Fc-part of the human IgG1.

SEQ ID NO: 19 represents the sequence of reverse primer used for PCR amplifying the Fc-part of the human IgG1.

10 SEQ ID NO: 20 represents the sequence of forward primer used for PCR amplifying the cDNA encoding the extracellular domain of CD70.

SEQ ID NO: 21 represents the sequence of reverse primer used for PCR amplifying the cDNA encoding the extracellular domain of CD70.

SEQ ID NO: 22 represents the sequence of forward primer used for PCR amplifying the minimal promoter of the c-fos gene.

15 SEQ ID NO: 23 represents the sequence of reverse primer used for PCR amplifying the minimal promoter of the c-fos gene.

SEQ ID NO: 24 represents the sequence of the sense oligonucleotide of the duplicated heat shock element.

20 SEQ ID NO: 25 represents the sequence of the anti-sense oligonucleotide of the duplicated heat shock element.

SEQ ID NO: 26 represents the sequence of PCR product fusing the coding region of CD70, Tenascin-C and human IgG1 Fc domain.

SEQ ID NO: 27 Represents the amino acid sequence of Fc-TNC-CD70.

SEQ ID NO: 28 Represents the amino acid sequence of FLAG-HIS-TEV-CD70.

25 SEQ ID NO: 29 represents the amino acid sequence of hCD27(CRD1-2)-Fc(hIgG1m3).

SEQ ID NO: 30 represents the amino acid sequence of hCD27(1-184)-Fc(mIgG2a).

SEQ ID NO: 31 represents the amino acid sequence of hCD27(1-64)-Fc(hIgG1m3).

30 SEQ ID NO: 32 represents the amino acid sequence of hCD27(1-184)-Fc(hIgG1m3).

SEQ ID NO: 33 represents the amino acid sequence of mCD27ECD- Fc(hIgG1m3).

SEQ ID NO: 34 represents the amino acid sequence of cynoCD27(1-184)-Fc(hIgG1m3).

35 SEQ ID NO: 35 represents the amino acid sequence of hCD27(106-184)-Fc(hIgG1m3,del).

SEQ ID NO: 36 represents the amino acid sequence of hCD27(CRD2-3)-Fc(hIgG1m3).

SEQ ID NO: 37 represents the amino acid sequence of hFc-TNC-mCD70-ECD.

5 SEQ ID NO: 38 represents the amino acid sequence of the variable heavy chain of Fab 2F2.

SEQ ID NO: 39 represents the amino acid sequence of the variable light chain of Fab 2F2.

SEQ ID NO: 40 represents the amino acid sequence of the variable heavy chain of Fab 5F24.

10 SEQ ID NO: 41 represents the amino acid sequence of the variable light chain of Fab 5F24.

SEQ ID NO: 42 represents the amino acid sequence of the variable heavy chain of Fab 5F32.

15 SEQ ID NO: 43 represents the amino acid sequence of the variable light chain of Fab 5F32.

SEQ ID NO: 44 represents the amino acid sequence of the variable heavy chain of Fab 10F13.

SEQ ID NO: 45 represents the amino acid sequence of the variable light chain of Fab 10F13.

20 SEQ ID NO: 46 represents the amino acid sequence of the variable heavy chain of Fab 10F31.

SEQ ID NO: 47 represents the amino acid sequence of the variable light chain of Fab 10F31.

25 SEQ ID NO: 48 represents the amino acid sequence of the variable heavy chain of Fab 11F26.

SEQ ID NO: 49 represents the amino acid sequence of the variable light chain of Fab 11F26.

SEQ ID NO: 50 represents the amino acid sequence of the variable heavy chain of Fab 0151.

30 SEQ ID NO: 51 represents the amino acid sequence of the variable light chain of Fab 0151.

SEQ ID NO: 52 represents the amino acid sequence of the variable heavy chain of Fab 0152.

35 SEQ ID NO: 53 represents the amino acid sequence of the variable light chain of Fab 0152.

SEQ ID NO: 54 represents the amino acid sequence of the variable heavy chain of Fab 0153.

SEQ ID NO: 55 represents the amino acid sequence of the variable light chain of Fab 0153.

5 SEQ ID NO: 56 represents the amino acid sequence of the variable heavy chain of Fab 0154.

SEQ ID NO: 57 represents the amino acid sequence of the variable light chain of Fab 0154.

10 SEQ ID NO: 58 represents the amino acid sequence of the variable heavy chain of Fab 0155.

SEQ ID NO: 59 represents the amino acid sequence of the variable light chain of Fab 0155.

DESCRIPTION

15 CD27 is a 55 kDa type I transmembrane glycoprotein belonging to the tumor necrosis factor receptor (TNFR) superfamily. TNFR family members are characterised by repetitive patterns of several cysteine-rich domains (CRDs). A pre-ligand-binding assembly domain (PLAD domain), located in CRD1, is common among the TNFR superfamily members and promotes ligand-independent multimers. CD27 is thought to be expressed as
20 a disulphide-linked dimer, but may form trimers of CD27-dimers or higher order multimers upon ligand binding as shown with other members of this receptor family.

The present invention relates to monovalent antibodies that are capable of specifically binding CD27 and antagonising its function by preventing signalling through CD27. Monovalent antibodies of the invention may be capable of preventing CD27 from
25 binding to its ligand, CD70 and thus block the CD27:CD70 interaction directly. Monovalent antibodies of the invention may, alternatively, be capable of antagonising CD27 without preventing CD70 binding. Monovalent antibodies of the invention may be capable of preventing CD27 receptors from cross-linking or multimerising, thereby blocking CD27 indirectly. Such cross-linking may be blocked by a monovalent antibody that specifically
30 binds the the pre-ligand-assembly domain, also known as "cysteine rich domain 1" (CRD1).

Monovalent antibodies according to the invention may thus interfere with CD27/CD70 mediated co-stimulation of CD4+ and CD8+ T cells. Monovalent antibodies according to the invention may modulate B-cell function or differentiation. They may, for example, be capable of inhibiting B-cell IgG production. Monovalent antibodies according to

the invention may modulate NK-cell function. Monovalent antibodies of the invention may reduce the release of any one of the following cytokines: GM-CSF, Mip-1 α , MCP-3.

Monovalent antibodies of the invention may be capable of binding both human CD27 and CD27 from another species than a human being. The term "CD27", as used
5 herein, thus encompasses any naturally occurring form of CD27 which may be derived from any suitable organism. For example, CD27 for use as described herein may be vertebrate CD27, such as mammalian CD27, such as CD27 from a primate (such as a human, a chimpanzee, a Cynomolgus monkey or a rhesus monkey); a rodent (such as a mouse or a rat), a lagomorph (such as a rabbit), or an artiodactyl (such a cow, sheep, pig or camel).
10 Preferably, the CD27 is human CD27 (SEQ ID NO: 1). The CD27 may be a mature form of CD27 such as a CD27 protein that has undergone post-translational processing within a suitable cell. Such a mature CD27 protein may, for example, be glycosylated. The CD27 may be a full length CD27 protein.

The term "antagonist" herein refers to a compound that does not provoke a
15 biological response upon binding to CD27, but blocks or dampens agonist-mediated responses. On the contrary, an "agonist" is a compound that induces a response upon binding to CD27. Upon binding to a target, an antagonist will reduce the cell response induced by an agonist, such as e.g. a natural ligand or a compound that functionally resembles the effects of the natural ligand.

The term "antibody" herein refers to a protein, derived from a germline
20 immunoglobulin sequence, which is capable of specifically binding to an antigen or a portion thereof. The term antibody includes full length antibodies of any class (or isotype), that is, IgA, IgD, IgE, IgG, IgM and/or IgY. An antibody that specifically binds to an antigen such as CD27, or portion thereof, may bind exclusively to that antigen, or portion thereof, or it may
25 bind to a limited number of homologous antigens, or portions thereof.

Natural full-length antibodies usually comprise at least four polypeptide chains: two heavy (H) chains and two light (L) chains that are connected by disulfide bonds. In some cases, natural antibodies comprise less than four chains, as in the case of the heavy chain only antibodies found in camelids (V_HH fragments) and the IgNARs found in Chondrichthyes.
30 One class of immunoglobulins of particular pharmaceutical interest are the IgGs. In humans, the IgG class may be sub-divided into 4 sub-classes IgG1, IgG2, IgG3 and IgG4, based on the sequence of their heavy chain constant regions. The light chains can be divided into two types, kappa and lambda chains based on differences in their sequence composition. IgG molecules are composed of two heavy chains, interlinked by two or more disulfide bonds,
35 and two light chains, each attached to a heavy chain by a disulfide bond. An IgG heavy chain

may comprise a heavy chain variable region (VH) and up to three heavy chain constant (CH) regions: CH1, CH2 and CH3. A light chain may comprise a light chain variable region (VL) and a light chain constant region (CL). VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs) or hyper-variable regions (HvRs), interspersed with regions that are more conserved, termed framework regions (FR). VH and VL regions are 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. The variable domains with the hypervariable regions of the heavy and light chains form a [binding] domain that is capable of interacting with an antigen, whilst the constant region of an antibody may mediate binding of the immunoglobulin to host tissues or factors, including, but not limited to various cells of the immune system (effector cells), Fc receptors and the first component (C1q) of the C1 complex of the classical complement system.

The term "monovalent antibody" herein refers to an antibody that has a single antigen recognition site which is specific for a target antigen.

Monovalent antibodies of the invention may be derived from monoclonal antibodies, which represent a set of unique heavy and light chain variable domain sequences expressed from a single B-cell or a clonal population of B cells. Monoclonal antibodies may be produced and purified using various methods that are known to the person skilled in the art. For example, antibodies may be produced from hybridoma cells or by B-cell expansion. Monoclonal antibodies or fragments thereof may be recombinantly expressed in mammalian or microbial expression systems, or by in-vitro translation. Monoclonal antibodies or fragments thereof may also be recombinantly expressed as cell surface bound molecules, by means of e.g. phage display, bacterial display, yeast display, mammalian cell display or ribosome or mRNA display. Once produced, the antibodies may be screened for binding to CD27.

Monovalent antibodies of the invention may be Fab fragments. "Fab fragments" of an antibody, including "Fab" and "F(ab')₂" fragments, are derived from antibodies by cleavage of the heavy chain in the hinge region on the N-terminal or C-terminal side of the hinge cysteine residues connecting the heavy chains of the antibody. A "Fab" fragment includes the variable and constant domains of the light chain and the variable domain and the first constant domain (CH1) of the heavy chain. "F(ab')₂" fragments comprise a pair of "Fab" fragments that are generally covalently linked by their hinge cysteines. A Fab' is formally derived from a F(ab')₂ fragment by cleavage of the hinge disulfide bonds connecting the heavy chains in the F(ab')₂. Other chemical couplings than disulfide linkages of antibody

fragments are also known in the art. A Fab fragment retains the ability of the parent antibody to bind to its antigen. F(ab')₂ fragments are capable of divalent binding, whereas Fab and Fab' fragments can bind monovalently. Generally, Fab fragments lack the constant CH2 and CH3 domains, i.e. the Fc part, where interaction with the Fc receptors would occur. Thus, Fab fragments are in general devoid of effector functions. Fab fragments may be produced by methods known in the art, either by enzymatic cleavage of an antibody, e.g. using papain to obtain the Fab or pepsin to obtain the F(ab')₂, Fab fragments including Fab, Fab', F(ab')₂ may be produced recombinantly using techniques that are well known to the person skilled in the art.

Monovalent antibodies of the invention may be Fv fragments. An "Fv" fragment is an antibody fragment that contains a complete antigen recognition and binding site, and generally comprises a dimer of one heavy and one light chain variable domain in association that can be covalent in nature, for example in a single chain variable domain fragment (scFv). It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six hypervariable regions or a subset thereof confer antigen binding specificity to the antibody. However, even a single variable domain comprising only three hypervariable regions specific for an antigen can retain the ability to recognise and bind antigen, although usually at a lower affinity than the entire binding site (Cai & Garen, *Proc. Natl. Acad. Sci. USA*, 93: 6280-6285, 1996). For example, naturally occurring camelid antibodies that only have a heavy chain variable domain (VHH) can bind antigen (Desmyter et al., *J. Biol. Chem.*, 277: 23645-23650, 2002; Bond et al., *J. Mol. Biol.* 2003; 332: 643-655).

Monovalent antibodies of the invention may be scFv fragments. "Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, where these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv, see Pluckthun, 1994, In: *The Pharmacology of Monoclonal Antibodies*, Vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315.

Monovalent antibodies of the invention may be linear antibodies. The expression "linear antibodies" refers to antibodies as described in Zapata et al., 1995, *Protein Eng.*, 8(10):1057-1062. Briefly, these antibodies contain a pair of tandem Fd segments (VH-CH1-VH-CH1) that, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Monovalent antibodies of the invention may be monobodies. The term “monobody” as used herein, refers to an antigen binding molecule with a heavy chain variable domain and no light chain variable domain. A monobody can bind to an antigen in the absence of light chains and typically has three hypervariable regions, for example CDRs designated CDRH1, CDRH2, and CDRH3. A heavy chain IgG monobody has two heavy chain antigen binding molecules connected by a disulfide bond. The heavy chain variable domain comprises one or more hypervariable regions, preferably a CDRH3 or HVL-H3 region.

Antibody fragments may be obtained using conventional recombinant or protein engineering techniques and the fragments can be screened for binding to CD27, or antagonistic function, in the same manner as intact antibodies.

Antibody fragments of the invention may be made by truncation, e.g. by removal of one or more amino acids from the N and/or C-terminal ends of a polypeptide. Fragments may also be generated by one or more internal deletions.

Monovalent antibodies of the invention may also be bispecific antibodies. The term “bispecific antibody” herein refers to an antibody that has two different antigen recognition sites, one of which is specific for the target antigen, CD27.

The term “bispecific antibody” herein refers to an antibody that has two unique antigen recognition sites, one of which enables the antibody to engage with CD27. The second binding site may or may not be functional. Multispecific antibodies are antibodies with the ability to engage two or more different antigens or two or more different epitopes on the same antigen. Multispecific antibodies thus comprise bispecific antibodies.

Bispecific antibodies in full length IgG format, mimicking natural antibodies, can be generated by fusion of two individual hybridomas to form a hybrid quadroma which produces a mixture of antibodies including a fraction of bispecific heterodimerising antibodies (Chelius D, et al.; MAbs. 2010 May-Jun; 2(3): 309–319). Bispecific heterodimerising antibodies may alternatively be produced by using recombinant technologies. Heterodimerisation can be also be achieved by engineering the dimerisation interface of the FC region to promote heterodimerisation. One example hereof are the so-called knob-in-hole mutations where sterically bulky sidechains (knobs) are introduced in one FC matched by sterically small sidechains (holes) on the opposite FC thereby creating steric complementarity promoting heterodimerisation. Other methods for engineered heterodimerisation FC interfaces are electrostatic complementarity, fusion to non-IgG heterodimerisation domains or utilising the natural Fab-arm exchange phenomenon of human IgG4 to control heterodimerisation. Examples of heterodimerised bispecific antibodies are well described in the literature, e.g. (Klein C, et al.; MAbs. 2012 Nov-Dec; 4(6): 653–663). Special attention has to be paid to the

light chains in heterodimeric antibodies. Correct pairing of LCs and HCs can be accomplished by the use of a common light chain. Again engineering of the LC/HC interface can be used to promote heterodimerisation or light cross-over engineering as in CrossMabs. In-vitro re-assembly under mildly reducing conditions of antibodies from 2 individual IgGs containing the right mutation can also be used to generate bispecifics. Also the natural Fab-arm exchange method is reported to ensure correct light chains pairing.

Multispecific antibody-based molecules may also be expressed recombinantly as fusion proteins combining the natural modules of IgGs to form multispecific and multivalent antibody derivatives as described in the literature. Examples of fusion antibodies are DVD-IgGs, IgG-scFV, Diabodies, DARTs etc (Kontermann, MAb. 2012 Mar-April 4(2): 182-197). Specific detection or purification tags, half-life extension polypeptides or other component can be incorporated in the fusion proteins. Additional non-IgG modalities may also be incorporated in the fusion proteins.

Multispecific antibody-based molecules may also be produced by chemical conjugation or coupling of individual full length IgGs or coupling of the fragments of IgGs to form multispecific and multivalent antibody derivatives as described in the literature. Examples of fusion antibodies are chemical coupled Fab'2, IgG-dimer etc (Kontermann, MAb. 2012 Mar-April 4(2): 182-197). Specific detection or purification tags, half-life extension molecules or other component can be incorporated in the conjugate proteins. Additional non-IgG polypeptide may also be incorporated in the fusion proteins.

Multispecific molecules may also be produced by combining recombinant and chemical methods including those described above.

Monovalent antibodies of the invention may be defined in terms of their complementarity-determining regions (CDRs). The term "complementarity-determining region" or "hypervariable region", when used herein, refers to the regions of an antibody in which amino acid residues involved in antigen binding are situated. The region of hypervariability or CDRs can be identified as the regions with the highest variability in amino acid alignments of antibody variable domains. Databases can be used for CDR identification such as the Kabat database, the CDRs e.g. being defined as comprising amino acid residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) of the light-chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy-chain variable domain (Kabat *et al.* (1991). Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Alternatively CDRs can be defined as those residues from a "hypervariable loop" (residues 26-33 (L1), 50-52 (L2) and 91-96 (L3) in the light-chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy-chain

variable domain; Chothia and Lesk, J. Mol. Biol 1987; 196: 901-917). Typically, the numbering of amino acid residues in this region is performed by the method described in Kabat *et al.*, *supra*. Phrases such as "Kabat position", "Kabat residue", and "according to Kabat" herein refer to this numbering system for heavy chain variable domains or light chain variable domains. Using the Kabat numbering system, the actual linear amino acid sequence of a peptide may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a framework (FR) or CDR of the variable domain. For example, a heavy chain variable domain may include amino acid insertions (residue 52a, 52b and 52c according to Kabat) after residue 52 of CDR H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The term "framework region" or "FR" residues refer to those VH or VL amino acid residues that are not within the CDRs, as defined herein.

An antibody of the invention may comprise a CDR region from one or more of the specific antibodies disclosed herein, such as a CDR region from within SEQ ID NOs: 38 to 59, as defined using Kabat or as defined herein using sequential numbering. An antibody of the invention may also comprise CDR regions from the specific antibodies disclosed herein, wherein at the most one out of five amino acids in any one of the CDR regions has been back-mutated using methods known to the person skilled in the art.

The term "antigen" (Ag) herein refers to the molecular entity used for immunisation of an immunocompetent vertebrate to produce the antibody that recognises the antigen. Herein, antigen is termed more broadly and is generally intended to include target molecules that are specifically recognised by the antibody, thus including fragments or mimics of the molecule used in the immunisation process, or other process, e.g. phage display, used for generating the antibody. In the context of the current invention, the antigen may be human CD27, a portion thereof or a fusion protein comprising a portion thereof, such as hCD7 fused to human IgG1 Fc or mouse IgG2a-Fc.

Antibodies that bind to the same antigen can be characterised with respect to their ability to bind to their common antigen simultaneously and may be subjected to "competition binding"/"binning". In the present context, the term "binning" refers to a method of grouping antibodies that bind to the same antigen. "Binning" of antibodies may be based on competition binding of two antibodies to their common antigen in assays based on standard techniques such as surface plasmon resonance (SPR), ELISA or flow cytometry.

An antibody's "bin" is defined using a reference antibody. If a second antibody is unable to bind to an antigen at the same time as the reference antibody, the second antibody is said to belong to the same "bin" as the reference antibody. In this case, the reference and the second antibody competitively bind the same part of an antigen and are coined

5 "competing antibodies". If a second antibody is capable of binding to an antigen at the same time as the reference antibody, the second antibody is said to belong to a separate "bin". In this case, the reference and the second antibody do not competitively bind the same part of an antigen and are coined "non-competing antibodies".

10 Antibody "binning" does not provide direct information about the epitope. Competing antibodies, i.e. antibodies belonging to the same "bin" may have identical epitopes, overlapping epitopes or even separate epitopes. The latter is the case if the reference antibody bound to its epitope on the antigen takes up the space required for the second antibody to contact its epitope on the antigen ("steric hindrance"). Non-competing antibodies usually have separate epitopes.

15 Monovalent antibodies of the invention may be capable of binding to the same bin as Fab 0151, Fab 0152, Fab 0153, Fab 0154, Fab 0155, Fab 0156, Fab 2F2, Fab 5F24, Fab 5F32, Fab 10F13, Fab 11F26 or Fab 10F31: they may compete with any one of these Fab fragments for binding to CD27. Monovalent antibodies of the invention may be capable of binding the PLAD domain (cysteine rich domain 1, CRD1) of CD27. Monovalent antibodies of
20 the invention may be capable of binding to the same bin as any one of Fabs 2F2, 5F24 and 0151: they may compete with Fab 2F2, Fab 5F24 or Fab 0151 for binding to CD27. Monovalent antibodies of the invention may be capable of binding cysteine rich domain 2 (CRD2) of CD27. Monovalent antibodies of the invention may be capable of binding cysteine rich domain 3 (CRD3) of CD27. Monovalent antibodies of the invention may also be able to
25 compete with CD70 for binding to CD27.

Monovalent antibodies of the invention, such as those capable of binding to the same bin as Fab 0151, Fab 0152, Fab 0153, Fab 0154, Fab 0155, Fab 0156, Fab 2F2, Fab 5F24, Fab 5F32, Fab 10F13, Fab 11F26 or Fab 10F31, may have one or both mutations V5Q and K45Q. Similarly any one of Fab 0151, Fab 0152, Fab 0153, Fab 0154, Fab 0155,
30 Fab 0156, Fab 2F2, Fab 5F24, Fab 5F32, Fab 10F13, Fab 11F26 or Fab 10F31 may comprise one or both back mutations V5Q and K45Q.

Monovalent antibodies of the invention and Fabs of the invention, including those comprising one or both back mutations V5Q and K45Q, may furthermore have a free cysteine introduced in one or both of N152C or G157C positions.

The monovalent antibodies of the invention, the Fabs of the invention, including those with one or both back mutations V5Q and K45Q, and those with a free cysteine in either or both N152C or G157C positions may be in unconjugated form or conjugated to a protractive group/half-life extending moiety.

5 Suitable embodiments include the humanized 5F24 fab, variants Hz5F24-V5Q-K45Q-N152C or Hz5F24-V5Q-K45Q-G157C of 0307-0000-0198, 0307-0000-0351, 0307-0000-0381, 0307-0000-0360, and 0307-0000-0382 unconjugated or conjugated to a protractive group/half-life extending moiety, such as to an Fc domain, transferrin, or an albumin binding peptide, preferably an albumin binding peptide.

10 The term "binding affinity" is herein used as a measure of the strength of a non-covalent interaction between two molecules, e.g. an antibody, or fragment thereof, and an antigen. The term "binding affinity" is used to describe monovalent interactions (intrinsic activity).

15 Binding affinity between two molecules, e.g. an antibody, or fragment thereof, and an antigen, through a monovalent interaction may be quantified by determining the equilibrium dissociation constant (K_D). In turn, K_D can be determined by measurement of the kinetics of complex formation and dissociation, e.g. by the SPR method. The rate constants corresponding to the association and the dissociation of a monovalent complex are referred to as the association rate constant k_a (or k_{on}) and dissociation rate constant k_d (or k_{off}),
20 respectively. K_D is related to k_a and k_d through the equation $K_D = k_d / k_a$.

 Following the above definition, binding affinities associated with different molecular interactions, such as comparison of the binding affinity of different antibodies for a given antigen, may be compared by comparison of the K_D values for the individual antibody/antigen complexes.

25 A monovalent antibody according to the current invention may be able to compete with another molecule, such as CD70 or another CD27 antibody, for binding to CD27. A monovalent antibody according to the current invention may be able to bind CD27 with a greater affinity than that of another molecule also capable of binding CD27. The ability of a monovalent antibody to compete with CD70 or another CD27 antibody for binding to CD27
30 may be assessed by determining and comparing the K_D value for the interactions of interest, such as a specific interaction between an antibody and an antigen, with that of the K_D value of an interaction not of interest. Typically, the K_D for the monovalent antibody with respect to the target will be 2-fold, preferably 5-fold, more preferably 10-fold less than K_D with respect to the other, non-target molecule such as unrelated material or accompanying material in the

environment. More preferably, the K_D will be 50-fold less, such as 100-fold less, or 200-fold less; even more preferably 500-fold less, such as 1,000-fold less, or 10,000-fold less.

The value of this dissociation constant can be determined directly by well-known methods. Standard assays to evaluate the binding ability of ligands such as antibodies
5 towards targets are known in the art and include, for example, ELISAs, Western blots, RIAs, and flow cytometry analysis. The binding kinetics and binding affinity of the antibody also can be assessed by standard assays known in the art, such as SPR.

A competitive binding assay can be conducted in which the binding of the antibody
10 to the target is compared to the binding of the target by another ligand of that target, such as another antibody.

A monovalent antibody of the invention may have a K_D for its target of $1 \times 10^{-8}M$ or less, or $1 \times 10^{-9}M$ or less, or $1 \times 10^{-10}M$ or less, $1 \times 10^{-11}M$ or less, or $1 \times 10^{-12}M$ or less. The K_D of an antibody of the current invention may be less than 0.8 nM, such as less than 0.7 nM, such as less than 0.6 nM, such as less than 0.5 nM, such as less than 0.4 nM, such as less
15 than 0.3 nM, such as less than 0.2 nM, such as less than 0.1 nM, such as less than 0.05 nM, such as less than 0.025 nM, such as less than 0.015 nM, such as between 0.015 nM and 0 nM.

Monovalent antibodies of the invention may be conjugated to a half-life extending moiety. The term "half-life extending moiety" is herein understood to refer to one or more
20 chemical groups attached to one or more amino acid side chain functionalities such as -SH, -OH, -COOH, -CONH₂, -NH₂, or one or more N- and/or O-glycan structures and that can increase *in vivo* circulatory half-life of a number of therapeutic proteins/peptides when conjugated to these proteins/peptides. Examples of protractive groups/half life extending moieties include: Biocompatible fatty acids and derivatives thereof, Hydroxy Alkyl Starch
25 (HAS) e.g. Hydroxy Ethyl Starch (HES), Poly Ethylene Glycol (PEG), Poly (Gly_x-Ser_y)_n (HAP), Hyaluronic acid (HA), Heparosan polymers (HEP), Phosphorylcholine-based polymers (PC polymer), Fleximers, Dextran, Poly-sialic acids (PSA), an Fc domain, Transferrin, Albumin, Elastin like peptides, XTEN polymers, Albumin binding peptides, a CTP peptide, and any combination thereof.

The most abundant protein component in circulating blood of mammalian species is
30 serum albumin, which is normally present at a concentration of approximately 3 to 4.5 grams per 100 mLs of whole blood. Serum albumin is a blood protein of approximately 65,000 daltons which has several important functions in the circulatory system. It functions as a transporter of a variety of organic molecules found in the blood, as the main transporter of
35 various metabolites such as fatty acids and bilirubin through the blood, and, owing to its

abundance, as an osmotic regulator of the circulating blood. Serum albumin has a half-life of more than one week, and one approach to increasing the plasma half-life of proteins has been to conjugate to the protein a moiety that binds to serum albumin. Albumin binding property may be determined as described in J. Med. Chem., 43, 1986, (2000) which is incorporated herein by reference.

Hydrophobic/lipophilic half-life extending moiety: The ligands according to the present invention are preferably conjugated with a half-life extending moiety that is largely lipophilic/hydrophobic in nature. In a preferred embodiment, the hydrophobic half-life extending moiety is capable of forming non-covalent complexes with albumin ("albumin binder"), thereby promoting the circulation of the derivative with the blood stream, and also having the effect of extending the time of action of the derivative. Thus, a preferred substituent, or moiety, as a whole may be referred to as an albumin binding moiety.

The half-life extending moiety is preferably at, or near, the opposite end of the albumin binding moiety as compared to its point of attachment to the CD27 according to the invention. The other portion of the albumin binding moiety, i.e. the portion in-between the half-life extending moiety and the point of attachment to the peptide, may be referred to as a linker moiety, linker, spacer, or the like. However, the presence of a linker is optional, and hence the albumin binding moiety may be identical to the half-life extending moiety.

In particular embodiments, the albumin binding moiety and/or the half-life extending moiety is lipophilic, and/or negatively charged at physiological pH (7.4).

The albumin binding moiety and/or the half life extending moiety may be covalently attached to an amino group of the peptide by conjugation chemistry such as by alkylation, acylation, or amide formation; or to a hydroxyl group, such as by esterification, alkylation; or to other groups through oxidation.

In a preferred embodiment, an active thiophilic derivative of the albumin binding moiety and/or the half life extending moiety is covalently linked to the thiol of a cysteine residue of the anti-CD27 Fab or Fab variant. Such thiophilic groups include, but are not limited to, maleimides, halo-maleimides, halides (especially haloacetyl), acryloyl-derivatives (eg. acrylates and acrylamides), vinylsulfones, reactive disulfide groups (eg. 2-pyridyl). Thus, the anti-CD27 Fab of the present invention is preferably linked to the albumin binding moiety through a thioether or disulfide bond.

Monovalent antibodies according to the present invention, such as e.g. Fab' fragments, may be designed to contain the naturally occurring cysteine residue from the heavy chain that forms part of one of the heavy chain sulphur bridges of an intact antibody. Cysteine residues can also be inserted by genetic engineering but there may be safety

advantages associated by employing naturally occurring cysteine residues for conjugation purposes.

In a preferred embodiment, an active ester of the albumin binding moiety and/or the hydrophobic half-life extending moiety is covalently linked to an amino group of a sialic acid residue or a sialic acid derivative, under formation of an amide bond (this process being referred to as acylation).

According to a highly preferred embodiment of the present invention, the albumin binding moiety is attached to the ligand via a glycan using enzymatic methods such as e.g. a method involving use of a sialyltransferase.

For the present purposes, the terms "albumin binding moiety", "half-life extending moiety", and "linker" include the un-reacted as well as the reacted forms of these molecules. Whether or not one or the other form is meant is clear from the context in which the term is used.

The term "fatty acid" refers to aliphatic monocarboxylic acids having from 4 to 28 carbon atoms, it is preferably unbranched, and/or even numbered, and it may be saturated or unsaturated

The term "fatty diacid" refers to fatty acids as defined above but with an additional carboxylic acid group in the omega position. Thus, fatty diacids are dicarboxylic acids.

The nomenclature is as is usual in the art, for example COOH, as well as HOOC-, refers to carboxy; -C₆H₄- to phenylen; CO, as well as -OC-, to carbonyl (O=C<); C₆H₅-O to phenoxy; and halide refers to the halogens -F, -Cl, -Br, -I, and -At.

In a preferred embodiment, the albumin binding moiety of the present invention comprises a fatty acyl group $-(CH_2)_n-CO-$, where $n = 1, 2, 3, \dots, 40$) or an omega-carboxy fatty acyl group $(HO_2C-(CH_2)_n-CO-$, where $n = 1, 2, 3, \dots, 40$) linked to the peptide or protein via a linker and a sialic acid residue or sialic acid derivative.

In a preferred embodiment, the albumin binding moiety of the present invention comprises a fatty acyl group $-(CH_2)_n-CO-$, where $n = 1, 2, 3, \dots, 40$) or an omega-carboxy fatty acyl group $(HO_2C-(CH_2)_n-CO-$, where $n = 1, 2, 3, \dots, 40$) linked to the peptide or protein via a linker and a cysteine residue. In a particular preferred embodiment, n is 16 or 18.

In another preferred embodiment, the albumin binding moiety of the present invention comprises a fatty acyl group of the type $R-(CH_2)_n-CO-$, where $n = 1, 2, 3, \dots, 40$, linked to the peptide or protein via a linker and a cysteine residue. R is a group comprising an acidic group, eg. tetrazol-5-yl or $-O-C_6H_4-COOH$. In a particular preferred embodiment, n is 14 or

Compounds having a $-(CH_2)_{12}-$ moiety are possible albumin binders in the context of this invention. If such a compound is attached to a protein or peptide and results in an increased plasma half life of said protein or peptide, it is understood that the albumin binder may contribute to the overall increase of plasma half life.

5 In a preferred embodiment the linker moiety, if present, has from 2 to 80 C-atoms, preferably from 5 to 70 C-atoms. In additional preferred embodiments, the linker moiety, if present, has from 4 to 20 hetero atoms, preferably from 2 to 40 hetero atoms, more preferably from 3 to 30 hetero atoms. Particularly preferred examples of hetero atoms are N-, and O-atoms. H-atoms are not hetero atoms.

10 In another embodiment, the linker comprises at least one OEG molecule, and/or at least one glutamic acid residue, or rather the corresponding radicals (OEG designates 8-amino-3,6-dioxaoctanic acid, i.e. this radical: $NH-(CH_2)_2-O-(CH_2)_2-O-CH_2-CO-$).

In one preferred embodiment, the linker moiety comprises a di-carboxamide moiety and the linker is linked to a cysteine residue through a thioether bond. In preferred examples,
15 the di-carboxamide moiety contains from 2-30 C-atoms, preferably 4-20 C-atoms, more preferably 4-10 C-atoms.

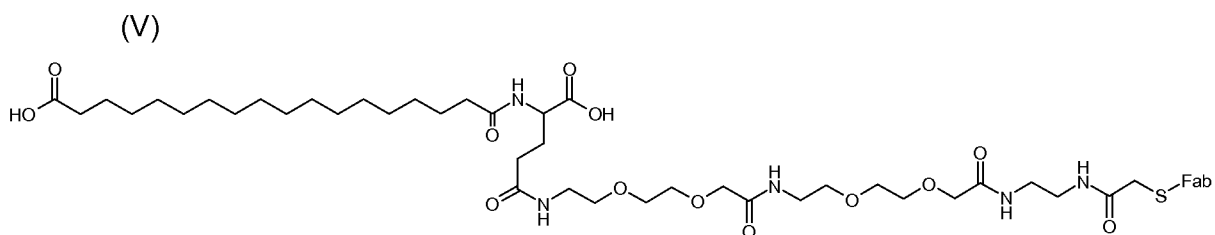
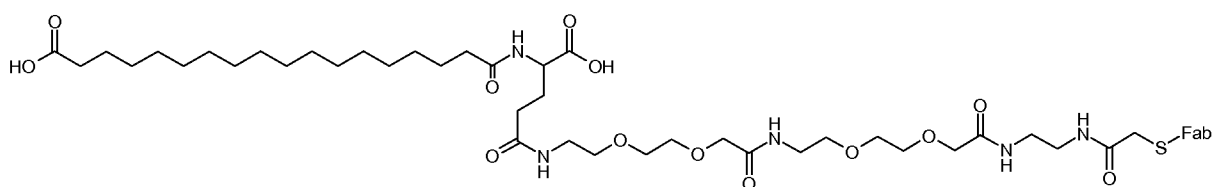
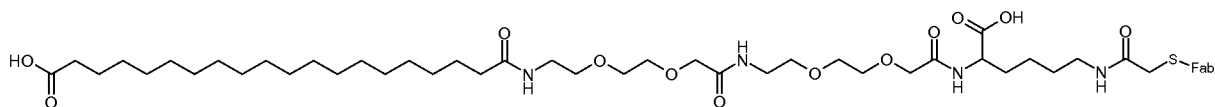
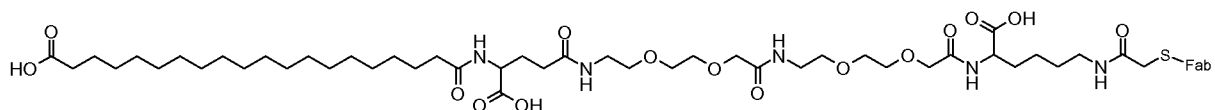
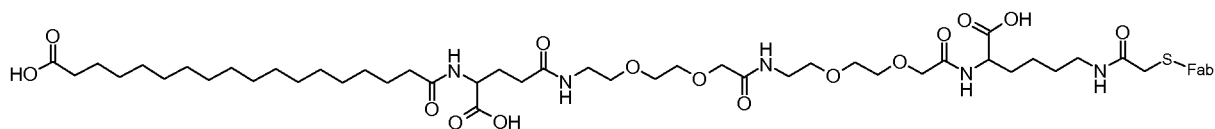
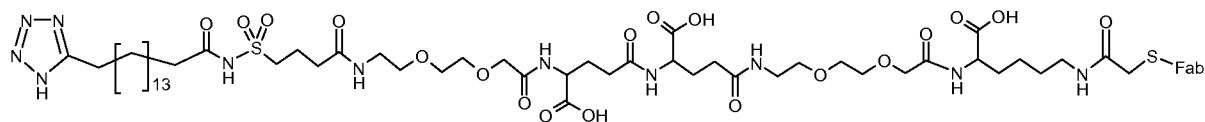
In one preferred embodiment, the linker moiety comprises a di-carboxamide moiety linked to a sialic acid residue by an amide bond. In preferred examples, the di-carboxyl residue has from 2-30 C-atoms, preferably 4-20 C-atoms, more preferably 4-
20 10 C-atoms. In additional preferred examples, the di-carboxyl residue has from 0- 10 hetero-atoms, preferably 0- 5 hetero-atoms.

In another preferred example, the linker moiety/spacer comprises a group containing both an amino and a distal carboxyl-group linked to a sialic acid residue by an amide bond through its distal carboxyl groups. In one preferred embodiment this group is an OEG group.
25 The term "hydrophilic spacer" as used herein means a spacer that separates a monovalent DR3 antibody/ligand according to the invention and an albumin binding residue with a chemical moiety which comprises at least 5 non-hydrogen atoms where 30-50% of these are either N or O. Preferably, the albumin binding residue is, via a hydrophilic spacer, linked to a Cys residue.

30 The amino acid glutamic acid (Glu) comprises two carboxylic acid groups. Its gamma-carboxy group is preferably used for forming an amide bond with an amino group of a sialic acid residue or a sialic acid derivative, or with an amino group of an OEG molecule, if present, or with the amino group of another Glu residue, if present. The amino group of Glu in turn forms an amide bond with the carboxy group of the half life extending moiety, or with
35 the carboxy group of an OEG molecule, if present, or with the gamma-carboxy group of

another Glu, if present. This way of inclusion of Glu is occasionally briefly referred to as "gamma-Glu".

In an interesting aspect of the invention the monovalent antibody may be conjugated with a lipophilic moiety. The lipophilic moiety suitably comprises a $-(CH_2)_n-CO-$ fatty acyl group, wherein n is 14-20, such as 15 or 16-18. The monovalent antibody may suitably be conjugated to a lipophilic moiety selected from the group consisting of formulas (I), (II), (III), (IV), (V), and (VI):



In another aspect, the present invention provides compositions and formulations comprising molecules of the invention, such as the monovalent antibodies described herein. For example, the invention provides a pharmaceutical composition that comprises one or

more monovalent CD27 antibodies of the invention, formulated together with a pharmaceutically acceptable carrier.

Accordingly, one object of the invention is to provide a pharmaceutical formulation comprising such a monovalent CD27 antibody which is present in a concentration from 0.25 mg/ml to 250 mg/ml, and wherein said formulation has a pH from 2.0 to 10.0. The formulation may further comprise one or more of a buffer system, a preservative, a tonicity agent, a chelating agent, a stabiliser, or a surfactant, as well as various combinations thereof. The use of preservatives, isotonic agents, chelating agents, stabilisers and surfactants in pharmaceutical compositions is well-known to the skilled person. Reference may be made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In one embodiment, the pharmaceutical formulation is an aqueous formulation. Such a formulation is typically a solution or a suspension, but may also include colloids, dispersions, emulsions, and multi-phase materials. The term "aqueous formulation" is defined as a formulation comprising at least 50% w/w water. Likewise, the term "aqueous solution" is defined as a solution comprising at least 50 % w/w water, and the term "aqueous suspension" is defined as a suspension comprising at least 50 %w/w water.

In another embodiment, the pharmaceutical formulation is a freeze-dried formulation, to which the physician or the patient adds solvents and/or diluents prior to use.

In a further aspect, the pharmaceutical formulation comprises an aqueous solution of such an antibody, and a buffer, wherein the antibody is present in a concentration from 1 mg/ml or above, and wherein said formulation has a pH from about 2.0 to about 10.0.

Monovalent CD27 antibodies and pharmaceutical formulations according to the invention may find use as medicaments, such as in the treatment of autoimmune diseases such as inflammatory bowel disease (IBD), Crohns disease (CD), ulcerative colitis (UC), irritable bowel syndrome, rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, systemic lupus erythematosus (SLE), lupus nephritis, type I diabetes, Grave's disease, multiple sclerosis (MS), autoimmune myocarditis, Kawasaki disease, coronary artery disease, chronic obstructive pulmonary disease, interstitial lung disease, autoimmune thyroiditis, scleroderma, systemic sclerosis, osteoarthritis, atopic dermatitis, vitiligo, graft versus host disease, Sjogrens's syndrome, autoimmune nephritis, Goodpasture's syndrome, chronic inflammatory demyelinating polyneuropathy, allergy, asthma and other autoimmune diseases that are a result of either acute or chronic inflammation. The term "treatment", as used herein, refers to the medical therapy of any human or other animal subject in need thereof. Said subject is expected to have undergone physical examination by a medical or veterinary medical practitioner, who has given a tentative or definitive diagnosis which would indicate that the

use of said treatment is beneficial to the health of said human or other animal subject. The timing and purpose of said treatment may vary from one individual to another, according to many factors, such as the status quo of the subject's health. Thus, said treatment may be prophylactic, palliative, symptomatic and/or curative. In terms of the present invention, prophylactic, palliative, symptomatic and/or curative treatments may represent separate aspects of the invention.

Inflammatory Bowel Disease (IBD) is a disease that may affect any part of the gastrointestinal tract from mouth to anus, causing a wide variety of symptoms. IBD primarily causes abdominal pain, diarrhea (which may be bloody), vomiting, or weight loss, but may also cause complications outside of the gastrointestinal tract such as skin rashes, arthritis, inflammation of the eye, fatigue, and lack of concentration. Patients with IBD can be divided into two major classes, those with ulcerative colitis (UC) and those with Crohn's disease (CD). While CD generally involves the ileum and colon, it can affect any region of the intestine but is often discontinuous (focused areas of disease spread throughout the intestine), UC always involves the rectum (colonic) and is more continuous. In CD, the inflammation is transmural, resulting in abscesses, fistulas and strictures, whereas in UC, the inflammation is typically confined to the mucosa. There is no known pharmaceutical or surgical cure for Crohn's disease, whereas some patients with UC can be cured by surgical removal of the colon. Treatment options are restricted to controlling symptoms, maintaining remission and preventing relapse. Efficacy in inflammatory bowel disease in the clinic may be measured as a reduction in the Crohn's Disease Activity Index (CDAI) score for CD which is scoring scale based on laboratory tests and a quality of life questionnaire. In animal models, efficacy is mostly measured by increase in weight and also a disease activity index (DAI), which is a combination of stool consistency, weight and blood in stool.

Psoriasis is a T-cell mediated inflammatory disorder of the skin that can cause considerable discomfort. It is a disease for which there is currently no cure and affects people of all ages. Although individuals with mild psoriasis can often control their disease with topical agents, more than one million patients worldwide require ultraviolet light treatments or systemic immunosuppressive therapy. Unfortunately, the inconvenience and risks of ultraviolet radiation and the toxicities of many therapies limit their long-term use. Moreover, patients usually have recurrence of psoriasis, and in some cases rebound shortly after stopping immunosuppressive therapy. A recently developed model of psoriasis based on the transfer of CD4+ T cells mimics many aspects of human psoriasis and therefore can be used to identify compounds suitable for use in treatment of psoriasis (Davenport et al., Internat. Immunopharmacol 2:653-672, 2002). Efficacy in this model is a measured by

reduction in skin pathology using a scoring system. Similarly, efficacy in patients is measured by a decrease in skin pathology.

Psoriatic arthritis is a type of inflammatory arthritis that occurs in a subset of patients with psoriasis. In these patients, the skin pathology/symptoms are accompanied by joint swelling, similar to that seen in rheumatoid arthritis. It features patchy, raised, red areas of skin inflammation with scaling. Psoriasis often affects the tips of the elbows and knees, the scalp, the navel and around the genital areas or anus. Approximately 10% of patients who have psoriasis also develop an associated inflammation of their joints.

Rheumatoid arthritis (RA) is a systemic disease that affects nearly if not all of the body and is one of the most common forms of arthritis. It is characterised by inflammation of the joint, which causes pain, stiffness, warmth, redness and swelling. This inflammation is a consequence of inflammatory cells invading the joints, and these inflammatory cells release enzymes that may digest bone and cartilage. As a result, this inflammation can lead to severe bone and cartilage damage and to joint deterioration and severe pain amongst other physiologic effects. The involved joint can lose its shape and alignment, resulting in pain and loss of movement.

There are several animal models for rheumatoid arthritis known in the art. For example, in the collagen-induced arthritis (CIA) model, mice develop an inflammatory arthritis that resembles human rheumatoid arthritis. Since CIA shares similar immunological and pathological features with RA, this makes it a suitable model for screening potential human anti-inflammatory compounds. Efficacy in this model is measured by decrease in joint swelling. Efficacy in RA in the clinic is measured by the ability to reduce symptoms in patients which is measured as a combination of joint swelling, erythrocyte sedimentation rate, C-reactive protein levels and levels of serum factors, such as anti-citrullinated protein antibodies.

Systemic lupus erythematosus (SLE) is an immune-complex related disorder characterised by chronic IgG antibody production directed at ubiquitous self-antigens, such as anti-dsDNA. The central mediator of disease in SLE is the production of auto-antibodies against self-proteins/tissues and the subsequent generation of immune-mediated inflammation. Antibodies either directly or indirectly mediate inflammation. Although T lymphocytes are not thought to directly cause disease, they are required for auto-antibody production. The effects of SLE are systemic (e.g., kidney, lung, musculoskeletal system, mucocutaneous, eye, central nervous system cardiovascular system, gastrointestinal tract, bone marrow, blood), rather than localised to a specific organ, although glomerulonephritis may result in some cases (i.e. lupus nephritis). Multiple chromosomal loci have been

associated with the disease and may contribute towards different aspects of the disease, such as anti-dsDNA antibodies and glomerulonephritis. Efficacy in SLE in human disease and in appropriate mouse models is measured by the ability of the therapeutic entity to decrease auto-antibodies (Eg: anti-dsDNA) and/or by decrease in renal pathology (enhanced kidney function), leading to amelioration of disease symptoms.

EMBODIMENTS

1. A monovalent antibody which is capable of specifically binding CD27 and reducing CD27 induced signalling, said antibody being capable of inhibiting B-cell IgG secretion by at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as 100%.
2. The monovalent antibody according to embodiment 1, which inhibits B-cell IgG secretion at a concentration of 0.05-0.5 µg/ml.
3. The monovalent antibody according to embodiment 1, said monovalent antibody having an IC₅₀ value that is less than 10nM, such as less than 9 nM, such as less than 8 nM, such as less than 7 nM, such as less than 6 nM, such as less than 5 nM, such as less than 4 nM, such as less than 3 nM, such as less than 2 nM, such as less than 1.5 nM, such as less than 1.0 nM, such as less than 0.5 nM.
4. The monovalent antibody according to any one of embodiments 1-3, which does not compete with CD70 for binding to CD27.
5. The monovalent antibody according to any one embodiments 1-3, wherein said antibody reduces CD27/CD70 mediated co-stimulation of CD4+ and/or CD8+ T cells by more than 50%.
6. The monovalent antibody according to embodiment 5, which reduces said stimulation of CD4+ and/or CD8+ T cells at a concentration of 0.1-1 µg/ml.
7. The monovalent antibody according to any one of embodiments 1-6, said antibody being capable of reducing CD70 induced GM-CSF release by 50%.

8. The monovalent antibody according to any one of embodiments 1-7, said antibody being capable of reducing Mip-1 α release.
9. The monovalent antibody according to any one of embodiments 1-8, said antibody being capable of reducing MCP-3 release.
10. The monovalent antibody according to any one of embodiments 7-9, which reduces said release at a concentration of 1-50 μ g/ml.
11. The monovalent antibody according to any one of embodiments 7-9, which reduces said release at a concentration of 2-10 μ g/ml.
12. The monovalent antibody according to embodiment 1, which is a Fab fragment.
13. The monovalent antibody according to embodiment 1, which is a bispecific antibody that is monovalent for CD27.
14. The monovalent antibody according to embodiment 13, the Fc domain of which has reduced effector functions.
15. The monovalent antibody according to embodiment 13, the Fc domain of which has increased stability.
16. The monovalent antibody according to any one of embodiments 1-15, which specifically binds CRD1 of CD27.
17. A monovalent antibody that is capable of specifically binding CD27, comprising a heavy chain having:
 - a CDRH1 sequence of amino acid residues 31 to 35 (DYAMH) of SEQ ID NO: 38, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or
 - a CDRH2 sequence of amino acid residues 50 to 66 (VISTYNGNTNYNQKLKG) of SEQ ID NO: 38, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 108 (DNGGTYGFTY) of SEQ ID NO: 38, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

- 5 18. The monovalent antibody according to embodiment 17, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (KASQDINSYLN) of SEQ ID NO: 39, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

- 10 a CDRL2 sequence of amino acid residues 50 to 56 (RANRLVD) of SEQ ID NO: 39, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

- 15 a CDRL3 sequence of amino acid residues 89 to 97 (LQYDEFPYT) of SEQ ID NO: 39, wherein one or two of these amino acid residues may be substituted with a different amino acid.

19. A monovalent antibody that is capable of specifically binding CD27, comprising a heavy chain having:

- 20 a CDRH1 sequence of amino acid residues 31 to 35 (NSWMN) of SEQ ID NO: 42, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 50 to 66 (RIYPGDGDTNYNGKFKG) of SEQ ID NO: 42, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

- 25 a CDRH3 sequence of amino acid residues 99 to 108 (GITATYYFDC) of SEQ ID NO: 42, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

- 30 20. The monovalent antibody according to embodiment 19, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (SASQGISNYLN) of SEQ ID NO: 43, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (YTSNLHS) of SEQ ID NO: 43, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

5 a CDRL3 sequence of amino acid residues 89 to 97 (QHYSKLPWT) of SEQ ID NO: 43, wherein one or two of these amino acid residues may be substituted with a different amino acid.

21. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

10 a CDRH1 sequence of amino acid residues 31 to 35 (DYGMA) of SEQ ID NO: 48, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 50 to 66 (FINNLAYSSYHADTVTG) of SEQ ID NO: 48, wherein one, two or three of these amino acids may be substituted by a
15 different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 106 (DRGGYFDY) of SEQ ID NO: 48, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

20 22. A monovalent antibody according to embodiment 21, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 33 (SASSSVSYMN) of SEQ ID NO: 49, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

25 a CDRL2 sequence of amino acid residues 49 to 56 (DTSNVAS) of SEQ ID NO: 49, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL3 sequence of amino acid residues 88 to 96 (QQWNSYPPT) of SEQ ID NO: 49, wherein one or two of these amino acid residues may be substituted with a different
30 amino acid.

23. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

a CDRH1 sequence of amino acid residues 31 to 35 (SYDIN) of SEQ ID NO: 46, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

5 a CDRH2 sequence of amino acid residues 50 to 66 (WIFPEDGSTTYNEKFKG) of SEQ ID NO: 46, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 104 (YPWFTY) of SEQ ID NO: 46, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

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24. The monovalent antibody according to embodiment 23, further comprising a light chain having:

15 a CDRL1 sequence of amino acid residues 24 to 39 (RSTKSLHTNGITYLY) of SEQ ID NO: 47, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 55 to 61 (QMSNLAS) of SEQ ID NO: 47, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

20 a CDRL3 sequence of amino acid residues 94 to 102 (AQNLELPLT) of SEQ ID NO: 47, wherein one or two of these amino acid residues may be substituted with a different amino acid.

25. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

25 a CDRH1 sequence of amino acid residues 31 to 35 (NFWMN) of SEQ ID NO: 40, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

30 a CDRH2 sequence of amino acid residues 50 to 66 (MIHPDSETRLNQKFKD) of SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 108 (LDNDYDALDY) of SEQ ID NO: 40, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

26. The monovalent antibody according to embodiment 25, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (HASQNFNVWLS) of SEQ ID NO: 41, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (KASNLHT) of SEQ ID NO: 41, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL3 sequence of amino acid residues 89 to 97 (QQGQSYPT) of SEQ ID NO: 41, wherein one or two of these amino acid residues may be substituted with a different amino acid.

27. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

a CDRH1 sequence of amino acid residues 31 to 35 (DYAIH) of SEQ ID NO: 44, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 50 to 66 (VISTYNGNTNNNQKFMG) of SEQ ID NO: 44, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 108 (DTGRYNWFF) of SEQ ID NO: 44, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

28. The monovalent antibody according to embodiment 27, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (KASQDINSYLS) of SEQ ID NO: 45, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (RANRLVE) of SEQ ID NO: 45, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL3 sequence of amino acid residues 89 to 97 (LQYDEFPYT) of SEQ ID NO: 45, wherein one or two of these amino acid residues may be substituted with a different amino acid.

29. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

a CDRH1 sequence of amino acid residues 30 to 34 (SNSIS) of SEQ ID NO: 50, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 49 to 64 (IIYFSGATNYANWAKG) of SEQ ID NO: 50, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 96 to 112 (DYDSYDAVSTNLYAFDP) of SEQ ID NO: 50, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

30. The monovalent antibody according to embodiment 29, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (QASEIIYANLA) of SEQ ID NO: 51, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (RASTLAS) of SEQ ID NO: 51, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL3 sequence of amino acid residues 89 to 100 (QQGYSSSNVDNP) of SEQ ID NO: 51, wherein one or two of these amino acid residues may be substituted with a different amino acid.

31. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

a CDRH1 sequence of amino acid residues 29 to 34 (SNAMS) of SEQ ID NO: 52, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 49 to 64 (TIYGSGSTYYATWAKG) of SEQ ID NO: 52, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 95 to 112 (DYDSYNNVGDTTLYAFDP) of SEQ ID NO: 52, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

- 5 32. The monovalent antibody according to embodiment 31, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (QASQNIYSNLA) of SEQ ID NO: 53, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

- 10 a CDRL2 sequence of amino acid residues 50 to 56 (RASYLAS) of SEQ ID NO: 53, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

- 15 a CDRL3 sequence of amino acid residues 89 to 100 (QQGYSCVNVDNV) of SEQ ID NO: 53, wherein one or two of these amino acid residues may be substituted with a different amino acid.

33. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

- 20 a CDRH1 sequence of amino acid residues 29 to 34 (RGYMS) of SEQ ID NO: 54, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 49 to 65 (YIEHDSNNTWYASWVKG) of SEQ ID NO: 54, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

- 25 a CDRH3 sequence of amino acid residues 96 to 107 (AYYDDYSNGFKL) of SEQ ID NO: 54, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

- 30 34. The monovalent antibody according to embodiment 33, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (QASQRISSWLA) of SEQ ID NO: 55, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (RTSTLEF) of SEQ ID NO: 55, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

5 a CDRL3 sequence of amino acid residues 89 to 99 (QCTYGSSYGSA) of SEQ ID NO: 55, wherein one or two of these amino acid residues may be substituted with a different amino acid.

35. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

10 a CDRH1 sequence of amino acid residues 30 to 34 (GYNMG) of SEQ ID NO: 56, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 49 to 64 (IIYPSGSTYYGNWAKG) of SEQ ID NO: 56, wherein one, two or three of these amino acids may be substituted by a
15 different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 95 to 110 (GEYGSAGYGYGHYFSF) of SEQ ID NO: 56, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

20 36. The monovalent antibody according to embodiment 35, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 25 to 35 (QASQTISNNLA) of SEQ ID NO: 54, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

25 a CDRL2 sequence of amino acid residues 51 to 57 (SASTLAS) of SEQ ID NO: 54, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL3 sequence of amino acid residues 90 to 103 (QSYHYTGKPDYGHA) of SEQ ID NO: 54, wherein one or two of these amino acid residues may be substituted with a
30 different amino acid.

37. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:

a CDRH1 sequence of amino acid residues 30 to 34 (SVAGS) of SEQ ID NO: 58, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

5 a CDRH2 sequence of amino acid residues 49 to 64 (TISSSGNTYYASWAKG) of SEQ ID NO: 58, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 95 to 105 (ASYGTYGYYNL) of SEQ ID NO: 58, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

10

38. A monovalent antibody according to embodiment 37, further comprising a light chain having:

15 a CDRL1 sequence of amino acid residues 25 to 35 (QASENIGNALA) of SEQ ID NO: 59, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 57 (AASDLAS) of SEQ ID NO: 59, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

20 a CDRL3 sequence of amino acid residues 90 to 103 (QSYVYIHSSNYGIT) of SEQ ID NO: 59, wherein one or two of these amino acid residues may be substituted with a different amino acid.

25 39. A monovalent antibody according to any one of embodiments 1-16 that is capable of specifically binding CD27, having a heavy chain and light chain of said antibody as defined in any one of SEQ ID NO's 60 to 72.

30 40. A monovalent antibody according to any one of embodiments 17-39 wherein the sequence of amino acid residues of SEQ ID NO's 38 to 72 comprise or is modified to comprise one or both backmutations V5Q in the Heavy Chain and K45Q in the Light Chain.

41. A monovalent antibody according to any one of embodiments 17-40 wherein a cysteine introduced in one or both of N152C or G157C positions.

42. A fusion protein or conjugate comprising the monovalent antibody according to any one of embodiments 1-41.
- 5 43. The fusion protein or conjugate according to embodiment 42, further comprising a half-life extending moiety.
44. The fusion protein or conjugate according to embodiment 43, wherein said half-life extending moiety is an albumin binding moiety.
- 10 45. The fusion protein or conjugate according to embodiment 44, wherein said albumin-binding moiety is bound to the C-terminal of the heavy chain fragment of the monovalent CD27 antibody.
- 15 46. The fusion protein or conjugate according to embodiment 43, wherein said monovalent antibody is conjugated to a half-life extending moiety via a glycan.
47. The monovalent antibodies according to any one of embodiments 17-41 in unconjugated form or conjugated to a protractive group/half-life extending moiety.
- 20 48. The monovalent antibodies according to embodiment 47, conjugated to a protractive group/half-life extending moiety selected from Fc domain, Transferrin, or an Albumin binding peptide.
- 25 49. A monovalent antibody according to any one of claims 1 to 16 that is capable of specifically binding CD27, wherein the heavy chain of said antibody comprises:
a CDRH1 sequence of amino acid residues 31 to 35 (NFWMN) of SEQ ID NO: 40, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or
a CDRH2 sequence of amino acid residues 50 to 66 (MIHPDSETRLNQKFKD) of
30 SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or
a CDRH3 sequence of amino acid residues 99 to 108 (LDNDYDALDY) of SEQ ID NO: 40, wherein one, two or three of these amino acid residues may be substituted by a different amino acid;

comprising a conjugated to a protractive group/half life extending moiety selected an albumin binding peptide.

50. The monovalent antibody according to any one of embodiments 1-41 and 47-49 or the fusion protein according to any one of embodiments 42-46 for use as a medicament.

51. The monovalent antibody according to any one of embodiments 1-41 and 47-49 , or the fusion protein or conjugate according to any one of embodiments 42-46, for use in the treatment of an autoimmune disease.

52. Use according to any one of embodiments 50-51, wherein said autoimmune disease is selected from the group consisting of rheumatoid arthritis, Crohn's disease, ulcerative colitis, systemic lupus erythematosus, psoriasis and psoriatic arthritis.

The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of all figures and all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

EXAMPLES

Example 1: Expression of Fusion Proteins

The coding regions of fusion proteins hCD27(CRD1-2)-Fc(hIgG1m3) (SEQ ID NO: 29), hCD27(1-184)-Fc(mIgG2a) (SEQ ID NO: 30), hCD27(1-64)-Fc(hIgG1m3) (SEQ ID NO: 31), hCD27(1-184)-Fc(hIgG1m3) (SEQ ID NO: 32), mCD27ECD- Fc(hIgG1m3) (SEQ ID NO: 33), cynoCD27(1-184)-Fc(hIgG1m3) (SEQ ID NO: 34), hCD27(106-184)-Fc(hIgG1m3,del) (SEQ ID NO: 35), and hCD27(CRD2-3)-Fc(hIgG1m3) (SEQ ID NO: 36) were cloned into the pJSV002 vector through the EcoR I and BamH I sites to construct eukaryotic expression plasmids. An extra Kozak sequence was introduced between EcoR I and start codon ATG to initiate translation process. Plasmid sequences were confirmed by sequencing.

Example 2: Protein expression

Plasmid DNA encoding the respective recombinant protein was transfected with 293fectinTM reagent (Invitrogen, cat. #12347) into FreestyleTM 293-F cells (Life Technologies, Cat. # R79007). For protein production, cells were grown in serum-free

FreeStyle 293 medium (Gibco, cat. #12338) containing 4 mM glutamine (Life Technologies, Cat. # 25030-024), 1% PLURONIC® F68 (Gibco, Cat. #24040-032) and penicillin-streptomycin antibiotics (Life Technologies, Cat. # 10378016) at 1×10^6 cells per ml and incubated with shaking for 5 days at 37 °C, 8% CO₂. Supernatants were collected on day 5 post transfection by centrifugation.

Example 3: Protein purification

The culture supernatant containing the target protein was harvested by centrifugation (15,000 rpm × 20 min, 4°C) and then cleared by the filtration with 0.2 µm cellulose nitrate membrane(Whatman, Cat. #7182-001). The cleared supernatant was applied to a Hitrap Mabselect SURE (GE Healthcare, Cat. #11-0034-94), followed by a 10 column volume wash with PBS pH7.4. The bound protein was then eluted with 50 mM Sodium Citrate pH3.3 and collected as 2 ml fractions into the glass tube with 200 ml 1 M Tris.Cl pH9.0. The fractions containing the target protein were pooled and concentrated to ~5 ml by Amicon ultra 15 centrifugal units (10,000 Da MWCO, Millipore, Cat. # UFC901024). The proteins were further polished and buffer-exchanged to PBS pH7.4 by a Hiload 26/60 Superdex 200 column (GE Healthcare, Cat. #28-9893-36). After concentrating, the final protein concentrations were determined by measuring 280 nm absorbance with NanoDrop 2000 (Thermo Scientific, Cat. #ND-2000). Protein purity was assessed by SDS-PAGE.

Example 4 – Expression of ligand and receptor

FLAG-tagged CD70

A FLAG-tagged version of CD70 was made for the FACS-based competition assay. CD70 extracellular domain coding sequence was PCR amplified using the two overlapping forward primers (SEQ ID NO: 10 og 11) and the CD70 reverse primer (SEQ ID NO: 12) and the IMAGE #3506629 DNA clone (GenBank Accession No. BC000725) as template. The primers contain sequence coding for FLAG-tag, HIS-tag and TEV cleavage site and the amplicon is a fusion between FLAG-tag, HIS-tag, TEV cleavage-site and CD70 extracellular domain (DNA sequence SEQ ID NO: 13 and protein sequence SEQ ID NO: 28). The PCR-product was cloned NheI / EcoRI into pTT5 vector (Durocher Y et al. , Nucleic Acids Res. 2002 Jan 15;30(2):E9) containing DNA sequence encoding the human CD33 signal peptide (DNA sequence SEQ ID NO: 14 and protein sequence SEQ ID NO: 15). The FLAG-HIS-TEV-CD70 was expressed transiently in Freestyle HEK293 cells (Invitrogen), transfected according to the manufacturer's protocol.

Fc-TNC-CD70

It has been reported the soluble CD27 ligand, CD70, is incapable of inducing any signal via the CD27 receptor (Wyzgol-A et al. The Journal of Immunology, 2009, 183: 1851-1861). Crosslinking of CD70 on the other hand can stimulate CD27 activation. We made a CD70 molecule fused to Tenascin-C, which is a trimerisation domain, in combination with a Fc-domain, which will form a dimer.

The Fc-domain part was made as PCR on the IMAGE #5440834 (Genbank Accession No. BC024289) template using the forward and reverse primers (SEQ ID NO: 18 and SEQ ID NO: 19) and the CD70 was made on IMAGE#3506629 template (Genbank Accession No. BC000725) with forward and reverse primers (SEQ ID NO: 20 and SEQ ID NO: 21). The resulting PCR-products were mixed and an overlapping PCR was performed using forward and reverse primers (SEQ ID NO: 18 and SEQ ID NO: 21). The final PCR-product (SEQ ID NO: 26) was cloned into pTT5 using EcoR I and Not I restriction enzymes. The Fc-TNC-CD70 protein (SEQ ID NO: 27) was expressed transiently in Freestyle HEK293 cells (Invitrogen), transfected according to the manufacturers protocol.

CD27/HEK293 cells

The coding region of CD27 (ref seq mRNA NM_001242.4) was also cloned BamHI/XhoI into pcDNA3.1(hygro)+ (Invitrogen Cat No. V875-20) and stably transfected into HEK-293 cells (ATCC CRL-1573) using the FuGene 6 transfection reagent (Roche Applied Science). Stable clones were selected by 100 microgram/ml Hygromycin B (Invitrogen) and isolation of single clones were performed by FACS sorting using an APC-Armenian hamster anti mouse CD27 antibody (BD Pharmingen Cat. No. 580691).

Example 5: Purification of recombinant human and murine CD70 proteins

Several recombinant epitope-tagged CD70 proteins were expressed in HEK293 cells and purified. They comprised the extracellular domain of CD70, fused to either an human IgG4 Fc domain or a epitope tag containing the FLAG peptide, a HIS tag and a TEV protease cleavage site. CD70 is the natural ligand for CD27 and the recombinant proteins were used to stimulate CD27 signalling in cellular assays and to test competition of anti-CD27 mAB and Fab for binding to CD27.

In case of the Fc fusion proteins (SEQ ID NO: 27; SEQ ID NO: 37), cell supernatants were loaded directly onto a column with Protein A affinity beads (MabSelect Sure; GE Healthcare, cat. #: 17-5438-01) followed by a washing step with 20mM Tris/HCl, pH 8.5. Bound proteins were eluted stepwise with 10 mM, 50 mM and 100mM

sodium formiate, pH 3.5. The pH of the eluted proteins was adjusted with 1 M Tris, pH 8.0. Eluted fractions were concentrated and shifted to PBS buffer by ultrafiltration using Amicon ultra 15 centrifugal units (10,000 Da MWCO, Millipore, Cat. # UFC901024). Gel filtration was carried out on a Superdex200 column (GE Healthcare) with PBS as buffer. Fractions of the main peak were pooled and concentrated by ultrafiltration.

FLAG-HIS-TEV-CD70 (SEQ ID NO: 28) protein was purified by Ni-NTA affinity chromatography. 15 mL Ni-NTA superflow (Qiagen,) was packed into a column and equilibrated with buffer A (5mM imidazol, 300mM NaCl , 5% Glycerol, pH 7,2). Cell supernatant was loaded directly on the column followed by a washing step with buffer A . Bound proteins were eluted stepwise with 10 %, 50 % and 100 % buffer B (300mM imidazol, 300mM NaCl, 5% glycerol, pH 7,2). The main portion of the protein eluted at 50 % B. These fractions were concentrated and shifted to PBS buffer by ultrafiltration using an Amicon ultra 15 centrifugal unit (10,000 Da MWCO, Millipore, Cat. # UFC901024). Gel filtration was carried out on a Superdex75 column (GE Healthcare) with PBS as buffer. Fractions of the main peak were pooled and concentrated by ultrafiltration.

All chromatography steps were performed with an Äkta Explorer FPLC instrument (GE Healthcare).

Example 6: Immunisation

RBF and NMRCF1 mice were immunised by injecting 50µg of soluble human CD27-Fc fusion protein (SEQ ID NO 20) in FCA subcutaneously followed by two injections with 20µg of the same fusion in Freunds Incomplete Adjuvans. New Zealand White rabbits were immunised by injecting 50 µg of soluble human CD27-Fc fusion protein (SEQ ID NO: 30) in FCA subcutaneously followed by two injections with 25 µg of the same protein in Freunds Incomplete Adjuvans.

We have used two different constructs of the extracellular domain (ECD) of human CD27: hCD27 fused to either human IgG1 Fc (Sino Biological Inc; cat no.: 10039-H03H) or fused to mouse IgG2a-Fc.

Example 7: Fusion and primary screen

High responder mice were boosted intravenously with 25 µg of the immunogen and sacrificed three days later. The spleen was removed aseptically and dispersed to a single cell suspension. Spleen cells were fused with the myeloma Fox cell line (RBF mice) or X63 (NMRCF1 mice) using electrofusion. After 10 days and one change of medium, supernatants were screened for binding to human CD27-Fc protein by ELISA and for binding to hCD27

expressing Hek293 cells either by imaging (FMAT or Celigo) or by flow cytometry. All selected clones were purified by affinity chromatography (Prot. A) before further characterisation.

5 *Selected clones are shown in Table 1:*

Clone	isotype	Antigen For screening	Mouse	Antigen For immunisation
M-CD27-2F2	IgG1	CD27-hFc (Sino Bio.)	#9316C, RBF strain	CD27-hFc (Sino Bio.)
M-CD27-5F2	IgG1	CD27-hFc (Sino Bio.)	#9343B RBF strain	CD27-mFc (SEQ ID NO: 30)
M-CD27-5F32	IgG1	CD27-hFc (Sino Bio.)	#9343B RBF strain	CD27-mFc (SEQ ID NO: 30)
M-CD27-10F13	IgG1	hCD27(1-184)- Fc(hIgG1m3) (SEQ ID NO: 32)	#9436D NMRCF1 strain	CD27-mFc (SEQ ID NO: 30)
M-CD27-10F31	IgG1	hCD27(1-184)- Fc(hIgG1m3) (SEQ ID NO: 32)	#9436D NMRCF1 strain	CD27-mFc (SEQ ID NO: 30)
M-CD27-11F26	IgG1	CD27-hFc (Sino Bio.)	#9412A RBF strain	CD27-mFc (SEQ ID NO: 30)

High responder rabbits were boosted intravenously with 50 µg of the immunogen and were sacrificed three days later. The spleen was removed aseptically and dispersed to a single cell suspension. Rabbit B cells expressing antibodies specific for human CD27 (1-184) Fc (IgG1) (SEQ ID 32) were identified and selected by fluorescence activated single cell sorting from a preparation of spleenocytes and were seeded monoclally in 384 well plates on a layer of irradiated murine EL4.B5 feeder cells and cultured for 7 days in medium supplemented with rabbit BAFF, IL-2, and PMA-activated splenic supernatant to support growth and survival of the single B-cell. Supernatants from propagated B cells were tested for presence of mCD27 and hCD27 specific IgG in a direct ELISA. B cells positive in both ELISAs were harvested and cDNA encoding heavy- and light chain were cloned and expressed in HEK293 cells as human and rabbit chimeric Fabs. Antigen-specificity of chimeric Fabs was confirmed and cross-reacting Fabs were further characterised.

Example 8: Cloning of rabbit anti-CD27 antibodies

Rabbit variable domain of heavy chain (VH) and variable domain of light chain (VL) were cloned from expanded rabbit B-cell cultures according to a generic setup.

RT-PCR on expanded B-cell cultures (RT-PCR, Qiagen, cat. No. 210212) was made by mixing 20 ul of PBS washed B-cell culture with a master mix containing RT-PCR and PCR primers. The primers used for VH and VL cloning were: pMskj1626 (5'-3') (SEQ ID NO: 2), PSqld1241 (5'-3'): (SEQ ID NO: 3), pMskj1630 (5'-3'): (SEQ ID NO: 4) and PSqld1243 (5'-3'): (SEQ ID NO: 5).

VH and VL PCR products were purified using magnetic beads (AMPure, Beckman Coulter, Cat. No. A63881). The PCR products were subcloned inframe into pTT based expression acceptor vectors using In-Fusion cloning (Clontech, Cat. No. 639650).

Generation of expression vectors for rabbit/human chimeric anti-CD27 Fab fragments

A series of CMV promotor-based based expression vectors (pTT vectors) were generated for transient expression of rabbit/human chimeric anti-CD27 antibody fragments in the HEK293-6E EBNA-based expression system developed by Yves Durocher (Durocher *et al.* Nucleic Acid Research, 2002). In addition to the CMV promotor, the pTT-based vectors contain a pMB1 origin, an EBV origin and the Amp resistance gene. The expression vectors used, pBF554 and pSL330, were designed to code for hCH1 (hIgG4) and hKappa respectively.

Sequencing was performed at MWG Biotech, Martinsried, Germany using sequencing primers anchoring in the plasmid backbone. Sequences were analyzed and annotated using the VectorNTI program. All kits and reagents were used according to the manufacturer's instructions.

Recombinant expression of Fab variants

The chimeric anti-CD27 variants were expressed transiently in HEK293-6E cells using the pTT-based Fab LC/HC expression vectors according to a generic antibody expression protocol. The following procedure describes the generic transfection protocol used for suspension adapted HEK293-6E cells.

Cell maintenance:

HEK293-6E cells were grown in suspension in FreeStyle™ 293 expression medium (Gibco) supplemented with 25 µg/ml Geneticin (Gibco), 0.1% v/v of the surfactant Pluronic F-

68 (Gibco) & 1% v/v Penicillin-Streptomycin (Gibco). Cells were cultured in Erlenmeyer shaker flasks in shaker incubators at 37°C, 8 % CO₂ and 125 rpm and maintained at cell densities between 0.1-1.5 x 10⁶ cells/ml.

5 *DNA Transfection:*

The cell density of cultures used for transfection was 0.9-2.0 x 10⁶ cells/ml.

A mix of 0.5 µg LC vector DNA + 0.5 µg HC vector DNA was used per ml cell culture.

10 The DNA was diluted in Opti-MEM media (Gibco) 30µl media/µg DNA, mixed and incubated at room temperature (23-25 °C) for 5 min.

293Fectin™ (Invitrogen) was used as transfection reagent at a concentration of 1µl per µg DNA.

The 293Fectin™ was diluted 30X in Opti-MEM media (Gibco), mixed and incubated at room temperature (23-25 °C) for 5 min.

15 The DNA and 293Fectin solutions were mixed and left to incubate at room temperature (23-25 °C) for 25 min.

The DNA-293Fectin mix was then added directly to the cell culture.

The transfected cell culture was transferred to a shaker incubator at 37°C, 8 % CO₂ and 125 rpm.

20 5 days post transfection, cell culture supernatants were harvested by centrifugation, followed by filtration through a 0.22 µm PES filter (Corning).

Quantitative analysis of antibody production was performed by Biolayer Interferometry directly on clarified cell culture supernatants using the FortéBio Octet system and protein A biosensors.

25

Example 9: Cloning of mouse anti-CD27 antibodies

Mouse heavy and light variable domain sequences were cloned from hybridomas. Total RNA was extracted from hybridoma cells using the RNeasy-Mini Kit from Qiagen and used as a template for cDNA synthesis. cDNA was synthesised in a 5'-RACE reaction using
30 the SMART™ RACE cDNA amplification kit from Clontech. Subsequent target amplification of VH and VL sequences was performed by PCR using Phusion Hot Start polymerase (Finnzymes) and the universal primer mix (UPM) included in the SMART™ RACE kit as forward primer. The reverse primers were pSqli_353 (5'-3') (VL, SEQ ID NO: 6) and pSqli_554 (5'-3') (VH, SEQ ID NO: 7).

PCR products were separated by gel electrophoresis, extracted using the GFX PCR DNA & Gel Band Purification Kit from GE Healthcare Bio-Sciences and cloned for sequencing using a Zero Blunt TOPO PCR Cloning Kit and chemically competent TOP10 *E. coli* (Invitrogen). Sequencing was performed at MWG Biotech, Martinsried Germany using either M13uni(-21)/M13rev(-29) sequencing primers. Sequences were analyzed and annotated using the VectorNTI program. All kits and reagents were used according to the manufacturer's instructions.

A single unique mouse VL and VH were identified (mKappa and mIgG1). VH and VL sequences are listed below, the leader peptide sequences are not included.

Generation of expression vectors for mouse/human chimeric anti-CD27 Fab fragment

A series of CMV promotor-based expression vectors (pTT vectors) were generated for transient expression of mouse/human chimeric anti-CD27 Fab fragment in the HEK293-6E EBNA-based expression system developed by Yves Durocher (Durocher *et al.* Nucleic Acid Research, 2002). In addition to the CMV promotor, the pTT-based vectors contain a pMB1 origin, an EBV origin and the Amp resistance gene.

Fab LC expression vector:

A pTT-based LC vector was generated for transient expression of chimeric anti-CD27 Fab fragment. Initially the region corresponding to the VL domain of anti-CD27 (earlier subclone) was PCR amplified from an original TOPO sequencing clone, using primers specific for the N- and C-terminal sequences. The sense primer contained a terminal HindIII restriction site for cloning purposes and a Kozak sequence (5'-GCCGCCACC-3') immediately upstream of the ATG start codon. The anti-sense primer contained an in-frame BsiWI restriction site in the VL/CL transition sequence. The generated PCR fragment was restriction digested, cloned into a linearised pTT-based vector containing the sequence for a human kappa CL domain and subsequently transformed into *E. coli* for selection. The sequence of the final construct was verified by DNA sequencing.

Fab HC expression vectors:

A pTT-based HC vector was generated for transient expression of a chimeric anti-CD27 Fab fragment. Initially the region corresponding to the VH domain of anti-CD27 (earlier subclone) was PCR amplified from an original TOPO sequencing clone, using primers specific for the N-terminal sequence and VH/CH transition sequence. The sense primer

contained a terminal HindIII restriction site for cloning purposes and a Kozak sequence (5'-GCCGCCACC-3') immediately upstream of the ATG start codon.

The generated VH domain PCR fragment was fused to hIgG4 Ch1 using overlap PCR. The IgG4 CH domain is terminated in the hinge region after the wild-type GPPC sequence motif, generating a truncated HC with a free C-terminal cysteine. Vector constructs were transformed into *E. coli* for selection. The sequence of the final construct was verified by DNA sequencing.

Recombinant expression of Fab variants

The chimeric anti-CD27 Fab variants were expressed transiently in HEK293-6E cells using the pTT-based LC/HC expression vectors according to a generic antibody expression protocol. The following procedure describes the generic transfection protocol used for suspension adapted HEK293-6E cells.

Cell maintenance:

HEK293-6E cells were grown in suspension in FreeStyle™ 293 expression medium (Gibco) supplemented with 25 µg/ml Geneticin (Gibco), 0.1% v/v of the surfactant Pluronic F-68 (Gibco) & 1% v/v Penicillin-Streptomycin (Gibco). Cells were cultured in Erlenmeyer shaker flasks in shaker incubators at 37°C, 8 % CO₂ and 125 rpm and maintained at cell densities between 0.1-1.5 x 10⁶ cells/ml.

DNA Transfection:

The cell density of cultures used for transfection was 0.9-2.0 x 10⁶ cells/ml. A mix of 0.5 µg LC vector DNA + 0.5 µg HC vector DNA was used per ml cell culture. The DNA was diluted in Opti-MEM media (Gibco) 30µl media/µg DNA, mixed and incubated at room temperature (23-25 °C) for 5 min. 293Fectin™ (Invitrogen) was used as transfection reagent at a concentration of 1µl per µg DNA. The 293Fectin™ was diluted 30X in Opti-MEM media (Gibco), mixed and incubated at room temperature (23-25 °C) for 5 min.

The DNA and 293Fectin solutions were mixed and left to incubate at room temperature (23-25 °C) for 25 min. The DNA-293Fectin mix was then added directly to the cell culture. The transfected cell culture was transferred to a shaker incubator at 37°C, 8 % CO₂ and 125 rpm. 5 days post transfection, cell culture supernatants were harvested by centrifugation, followed by filtration through a 0.22 µm PES filter (Corning). Quantitative analysis of antibody production was performed by Biolayer Interferometry directly on clarified cell culture supernatants using the FortéBio Octet system and protein A biosensors.

Example 10: Generation of Fab fragments by papain cleavage of mAbs

Fab fragments were generated by cleavage of mAb using the Pierce Fab Preparation Kit (Thermo Scientific, cat. # 44985) according to manufacturer's instructions.

5 The cleaved mAb were passed through a 1 mL column with MabSelect Sure (GE Healthcare, cat.#: 17-5438-01) to remove the Fc parts and incompletely processed material. The Fab fragments were recovered from the flow-through. In some cases, gel filtration with a Superdex200 column (GE Healthcare) and PBS as buffer was performed with the Fab fragments in order to remove high molecular weight forms.

Example 11: Purification of recombinant Fab fragments

Recombinant Fab fragments were purified by chromatography steps using an Äkta Explorer FPLC system (GE Healthcare). Chimeric Fab fragments, containing the constant part of human kappa immunoglobulin light chain, were purified by affinity purification with
15 KappaSelect (GE Healthcare, cat.# 17-5458-01). Cell supernatants were loaded directly to a KappaSelect column, which was subsequently washed with PBS. Bound Fab fragments were eluted with 20mM sodium formate, pH 3.5. The pH of the eluted proteins was adjusted with 1 M Tris, pH 8.0. Polishing of the Fab and a buffer shift was performed with gel filtration carried out on a Superdex75 column (GE Healthcare) with PBS as buffer.

Example 12: Binding of antibodies and Fab fragments to different domains of CD27

The extracellular moiety of CD27 is located at the amino terminal portion of CD27 and is comprised of 3 TNF receptor cysteine-rich domains (CRD). Several fusion proteins
25 were expressed and purified which contained parts of the extracellular domain of CD27 fused to the Fc domain of a human IgG4. e.g. CRD1; CRD1+2; CRD2+3 and CRD3. Binding of Fab fragments and monoclonal antibodies to these proteins was measured by surface plasmon resonance (SPR), in order to distinguish between different classes of binders.

The experiments were performed on a Biacore T200 instrument (GE Healthcare)
30 and analysed with the Biacore T200 Evaluation Software. Anti-human IgG antibodies (Human Antibody Capture Kit from GE Healthcare; cat.# BR-1008-39) were immobilised on all flow cells of a CM5 sensor chip (GE Healthcare; cat.# BR-1000-12) according to manufacturer's instructions, with the exception of using an antibody concentration of 12.5 µg/mL for the immobilisation. Binding experiments were carried out with HBS-EP+ (GE
35 Healthcare; cat.# BR-1006-69) containing 0.1 % bovine serum albumin as running buffer.

The different DR3-Fc proteins were captured at densities between 50 and 110 RU. Monoclonal Ab and Fab fragments were diluted to 5-10 µg/mL and injected for 240 s at flow rates of 30 µL/min to measure binding. The chip surface was regenerated by 2 injections of 3 M MgCl₂.

- 5 Fab fragments fell into different classes, binding to different CRD domains, as shown in Table 2, below.

Table 2: Binding of mAb and Fab fragments to CD27-Fc fusion proteins, containing different CRD domains, was measured by SPR. +: binding; (+): very weak binding; -: no binding

Ligand mAb/Fab	hCD27(CRD1) - Fc (SEQ ID NO 31)	hCD27(CRD1-2) - hFc (SEQ ID NO: 29)	hCD27(CRD2-3) - hFc (SEQ ID NO: 36)	hCD27(CRD3) - hFc (SEQ ID NO: 35)	hCD27(1-184) - hFc (SEQ ID NO: 33)
mAb 2F2	-	-	+	+	+
mAb 5F24	(+)	(+)	-	-	+
mAb 5F32	-	(+)	+	(+)	+
mAb10F13	-	-	+	+	+
Fab 0151	-	(+)	-	(+)	+

These data indicate that mAb 2F2 and mAb 10F13 both bind to the CRD3 domain. mAb 5F32 binds only strongly the ligand containing both CRD2+3 domains, but only very weakly to CRD1+2 and CRD3, indicating that its epitope comprises parts of CRD2 & 3. mAb 5F24 seems to bind to CRD1, however the binding response was very weak and the data are therefore not conclusive. Fab 0151 had weak binding responses for proteins containing CRD1+2 and CRD3, suggesting that its epitope requires the presence of all 3 CRD for efficient binding. All tested mAb and Fab bound well to hCD27(1-184)-Fc containing all 3 CRD.

Example 13: Measurement of binding kinetics for anti-CD27 Fab fragments by SPR

In order to select high affinity binders and to rank the different Fab fragments, the binding constants of Fab binding to different human and cynomolgus CD27-Fc fusion proteins was measured by surface plasmon resonance.

The experiments were performed on a Biacore T200 instrument (GE Healthcare) and analysed with the Biacore T200 Evaluation Software. Anti-human IgG antibodies (Human Antibody Capture Kit from GE Healthcare; cat.# BR-1008-39) were immobilised on all flow cells of a CM5 sensor chip (GE Healthcare; cat.# BR-1000-12) according to manufacturer's instructions, with the exception of using an antibody concentration of 12.5 µg/mL for the immobilisation. Kinetic measurements were carried out with HBS-EP+ (GE Healthcare; cat.# BR-1006-69) containing 0.1 % human serum albumin as running buffer. Human CD27(1-184)-Fc (SEQ ID NO: 32) or cynomolgus CD27(1-184)-hFC (SEQ ID NO: 34) was captured on the chip at low surface densities between 15 and 60 RU. Fab fragments were diluted to a number of different concentrations between 90 and 0.37 nM. In cases where Fab fragments were generated by papain cleavage of mAb, the sequence and therefore MW and extinction coefficient were not known. The approximate concentration was therefore determined by measuring the absorbance at 280 nm and setting the MW to 48 kDa and the extinction coefficient to 1.4. Fab fragments were injected for 360 s, followed by a 900 s dissociation phase. Binding curves were measured at 25 °C with a flow rate between 30 and 60 µL/min. Regeneration of the chip surface was performed with 3 M CaCl₂ for 2 x 20 s. The raw data were double referenced by subtraction of the signals from a reference flow cell without captured ligand and a buffer blank. Determination of the kinetic parameters was performed by fitting with a 1:1 binding model. Kinetic parameters were calculated and are stated in Tables 3 and 4, below. Several Fab fragments were identified, which bound with sub-nanomolar affinities to human CD27(1-184)-Fc (SEQ ID NO: 32). Binding to cynomolgus CD27(1-184)-Fc (SEQ ID NO: 34) was generally weaker than to the human homologue.

Table 3: Binding kinetics of Fab fragments to human CD27(1-184)-Fc (SEQ ID NO: 32) were determined by SPR. Kinetic parameters are shown in the table.

Analyte	Ligand	k_a [1/Ms]	SE(k_a)	k_d [1/s]	SE(k_d)	K_D [M]	Rmax (RU)
Fab 8F3	hCD27(1-184)Fc	6.72E+05	7.00E+02	1.32E-03	9.80E-07	1.97E-09	12.7
Fab 8F4	hCD27(1-184)Fc	1.22E+06	3.70E+03	1.35E-03	3.00E-06	1.10E-09	9.5
Fab 8F7	hCD27(1-184)Fc	9.98E+05	4.90E+03	4.02E-03	1.90E-05	4.03E-09	11.8
Fab 8F8	hCD27(1-184)Fc	1.08E+06	4.30E+02	5.81E-04	3.10E-07	5.39E-10	9.4

Fab 5F10	hCD27(1-184)Fc	3.71E+05	7.10E+02	0.001417	2.80E-06	3.82E-09	18.7
Fab 5F17	hCD27(1-184)Fc	2.14E+05	5.80E+02	0.004333	5.60E-06	2.02E-08	24.6
Fab 5F30	hCD27(1-184)Fc	1.25E+05	5.10E+02	0.002403	4.50E-06	1.92E-08	16.7
Fab 5F32	hCD27(1-184)Fc	5.84E+05	2.70E+02	1.68E-04	5.30E-07	2.88E-10	21.4
Fab 5F19	hCD27(1-184)Fc	6.31E+04	1.20E+03	0.003653	4.90E-05	5.79E-08	16.5
Fab 5F21	hCD27(1-184)Fc	2.10E+05	6.00E+02	0.003864	8.50E-06	1.84E-08	20.8
Fab 5F24	hCD27(1-184)Fc	5.39E+05	2.60E+02	2.09E-05	5.70E-07	3.89E-11	14.7
Fab 5F34	hCD27(1-184)Fc	4.50E+05	1.40E+03	6.26E-04	1.40E-06	1.39E-09	14.3
Fab 2F2	hCD27(1-184)Fc	7.02E+05	3.20E+02	1.59E-04	3.50E-07	2.27E-10	7.3
Fab 10F13	hCD27(1-184)Fc	1.57E+05	9.10E+01	7.66E-05	4.40E-07	4.87E-10	7.3
Fab 10F31	hCD27(1-184)Fc	5.67E+05	1.50E+03	1.36E-03	2.90E-06	2.39E-09	4.8

Table 4: Binding kinetics of Fab fragments to cynomolgus CD27(1-184)-Fc (SEQ ID NO: 34) was determined by SPR. Kinetic parameters are shown in the table.

Analyte	Ligand	k_a [1/Ms]	SE(k_a)	k_d [1/s]	SE(k_d)	K_D [M]	Rmax (RU)
Fab 2F2	cynoCD27(1-184)Fc	6.43E+05	5.60E+02	7.70E-04	4.60E-07	1.20E-09	19.3
Fab 10F13	cynoCD27(1-184)Fc	6.18E+04	1.40E+02	1.62E-03	2.20E-06	2.62E-08	18.0

5

Example 14: Competition assay

A competition assay was performed with different mAb and Fab fragments in order to determine if they belong to the same epitope bins.

The experiments were performed on a Biacore T200 instrument (GE Healthcare). Anti-human IgG antibodies (Human Antibody Capture Kit from GE Healthcare; cat.# BR-1008-39) were immobilised on all flow cells of a CM5 sensor chip (GE Healthcare; cat.# BR-1000-12) according to manufacturer's instructions, with the exception of using an antibody concentration of 12.5 µg/mL for the immobilisation. Human CD27(1-184)-Fc (SEQ ID NO: 32) was captured at low surface densities. Murine Fab fragments or mAb, which are not recognised by the capture antibody, were diluted to 10 or 15 µg/mL and injected using a dual injection, where 2 mAb or Fab are injected subsequently. It is important that the first antibody or Fab fragment reaches equilibrium and saturates binding to the captured ligands. Under these conditions, the second antibody can only bind if it belongs to a different epitope bin and does not compete with the first mAb or Fab for binding to the ligand. The chip surface was then regenerated by 2 injections of 3 M CaCl₂ for 2 x 20 s. Binding responses of the Fab fragments were analysed with Scrubber2 (BioLogic Software, Australia). Fab fragments, which did not bind simultaneously to CD27-Fc, were considered to belong to the same epitope bin.

1. Ab	mAb	mAb	mAb	mAb	Fab	Fab	Mab	Ab	Fab	Ab
2. AB	2F2	5F24	5F32	10F13	LG. 7F9	LG.3A1 0	382	89258	0151	1091
mAb 2F2	-	n.d.	n.d.	n.d.	+	+	-	-	n.d.	+
mAb 5F24	n.d.	-	n.d.	n.d.	+	+	+	+	n.d.	-
mAb 5F32	n.d.	n.d.	-	n.d.	+	+	-	-	n.d.	+
mAb 10F13	n.d.	n.d.	n.d.	-	+	+	-	-	n.d.	n.d.
Fab LG. 7F9	+	+	+	+	-	n.d.	n.d.	n.d.	-	n.d.
Fab LG.3A10	+	+	+	+	n.d.	-	n.d.	n.d.	-	n.d.
Mab382	-	+	(+)	-	n.d.	n.d.	-	n.d.	+	n.d.
ab89258	-	+	(+)	-	n.d.	n.d.	n.d.	-	+	n.d.
Fab 0151	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	+
ab1091	+	-	+	n.d.	n.d.	n.d.	n.d.	n.d.	+	-

Table 5: Epitope binning of commercial and in-house inhibiting anti-CD27 mAb & Fab.

MAB382 is from R&D Systems (cat.# MAB382, clone 57703), ab89258 is from Abcam (cat.# ab89258, clone MM0165-7H21). Fab LG.7F9 and Fab LG3A10 were generated by papain cleavage of the following 2 mAb (eBioscience, cat.# 16-0272-85, clone LG.3A10 and eBioscience, cat.# 16-0271-85, clone LG.7F9) and ab1091 is from Abcam (cat.# ab1091, clone LT27)

*(+): weaker binding than for other combinations with mAb 5F32 as 2. mAb
n.d.: not determined.*

The data in Table 5 demonstrate that mAb 2F2 and mAb 10F13 belong to the same epitope bin as the commercial antibodies MAB382 and ab89258, corroborated by the fact that they bind to CRD3 of CD27. Fab fragment 0151 and the commercial Fab fragments of mAb LG.7F9 and LG.3A10 seem to belong to the same epitope bin. Monoclonal Ab 5F32 seems to have a partially overlapping epitope with MAB382 and ab89258. This is in agreement with the binding of mAb 5F32 to CRD2+3, indicating that its epitope involves both CRDs. The presence of either MAB382 or ab89258 prevented binding of mAb 5F32, whereas both commercial antibodies could bind weakly in the presence of mAb 5F32. Together these data suggest that the biggest contribution to mAb 5F32 binding stems from interaction with CRD2. Monoclonal Ab 5F24 did compete with the commercial monoclonal Ab ab1091, indicating that they belong to the same epitope bin.

Example 15: Cellular binding assay

Binding of anti-hCD27 mAbs and Fabs to primary human CD4⁺T cells was analysed by flow cytometry. Anti-hCD27 mAbs and Fabs were titrated and incubated with cells and binding detected by a secondary APC-labeled antibody and signal measured by flow cytometry. PBMCs were purified from buffy coat from Rigshospitalet, Denmark. The buffy coat was transferred to a sterile flask (V=50 ml) and 100 ml RPMI 1640 + Glutamax (cat. no. 61870, Gibco Life Technologies, Nærum, Denmark) was added. 12.5 ml Ficoll-Paque Plus (Cat. no. 17-1440-02, GE Healthcare, Life Sciences) was added to six 50 ml tubes. 25 ml diluted buffy coat was carefully added on top of Ficoll-Paque by slowly pipetting down the side of the tube. The tubes were centrifuged 30 min, 950 x g, without brake. The interphase was transferred to new tube with pasteur pipettes by pooling the six tubes to three tubes. RPMI was added up to 45 ml. The tubes were centrifuged 10 min at 300 x g. The supernatant was discarded and cells were resuspended in a few ml of RPMI and pooled to two tubes. RPMI was added up to 45 ml. The tubes were centrifuged 10 min at 300 x g. The supernatant was discarded and cells were resuspended in 30 ml of RPMI in one tube and

counted by Cedex HiRes (Innovatis, Roche Diagnostics, Hvidovre, Denmark). Viability of PBMCs was >98%. The cells were resuspended in FACS buffer (DPBS with 2 % Fetal Calf Serum (FCS, Cat no. 10091, Gibco, Life Technologies, Nærum, Denmark) and 0,1% Azide (Cat no. AMPQ52300.0500, Ampliqon, Skovlunde, Denmark)) at 2×10^6 c/ml and kept on ice.

In a 96 well round bottom plate (Cat. no. 650201, Greiner Bio-one, Frickenhausen, Germany) 50 μ L/w aCD27 mAb or Fab was added at two times the desired final concentration (the final concentration used was 10 μ g/ml, 1.0 μ g/ml, 0.1 μ g/ml, 0.01 μ g/ml, 0.001 μ g/ml, 0.0001 μ g/ml and 0 μ g/ml). 25 μ L/w cell suspension was added containing either 50.000 c/well HEK293/hCD27 cells or PBMCs. 25 μ L/w FACS buffer was added. The cells incubated 1h on ice. Relevant isotype was included. 150 μ L/w FACS buffer was added, the plate was centrifuged 300 g, 5 min. The supernatant was discarded. 200 μ L/w FACS buffer was added and the plate was centrifuged at 300 g, 5 min. Finally the cells were resuspended in 50 μ L/w secondary Ab (either Anti-mu IgG (H+L)-PE (Cat. no. 115-116-146, Jackson ImmunoResearch, Suffolk, UK) or Anti-hu IgG (H+L)-PE (Cat. no.109-116-127, Jackson ImmunoResearch, Suffolk, UK) diluted 1:200 in FACS buffer. Anti-CD4-APC (Cat. no. 555349, BD Biosciences, NJ, USA) was added diluted 1:40 together with the secondary Ab. The cells were Incubated 30 min on ice. The washing procedure was repeated. Cells were resuspended in 100 μ L PBS. A dead/live marker was included in selected wells in order to ensure gating on living cells (7AAD, Cat. no. 559925, BD Biosciences, NJ, USA). The cells were analysed by flow cytometry on FACS Canto (BD Biosciences, NJ, USA). Cells alone, secondary antibody alone and relevant isotype controls were included as controls.

Antibodies and Fabs were titrated from 10 μ g/ml to 0.0001 μ g/ml in a 10-fold dilution row and plotted by using GraphPad Prism version 6. Curves were fitted by using a nonlinear four parameter fit. The concentration in nM that resulted in 50% of maximum binding to human CD4+ T cells was calculated from the curves (Table 6).

Table 6: EC50 values of a-hCD27 Fabs. Binding to human CD4+ T cells

Clone ID	Enzymatically cleaved Fab	Rec. hIgG4 chimeric Fab
2F2	0.88 nM	0.54 nM
5F24	0.21 nM	
5F32	0.30 nM	
10F13	2.38 nM	
10F31	5.66 nM	

11F26	Poor binding	
Rb F1		1.48 nM
Rb F13		4.85 nM
Rb F14		4.55 nM
Rb F15		10.26 nM
Rb F2		7.27 nM
Rb F22		19.32 nM

Example 16: Ligand competition assay

Anti-hCD27 mAbs and Fabs that bind cellular hCD27 were tested for the ability to compete with binding of FLAG-tagged recombinant human (rh)-CD70 to HEK293/hCD27 cells, where binding of CD70-FLAG was monitored by a secondary anti-FLAG mAb.

HEK293 cells were detached by washing with PBS and adding Versene (Cat. no. 15040033, Gibco, Life Technologies, Nærum, Denmark) and then incubated at 37°C for 10 min. The cells were centrifuged at 300 g, 10 min and resuspended in FACS buffer

(Dulbecco's Phosphate-Buffered Saline (DPBS-Ca²⁺/-Mg²⁺, Cat no. 14190-169, Gibco, Life Technologies, Nærum, Denmark) with 2 % Fetal Calf Serum (FCS, Cat no. 10091, Gibco, Life Technologies, Nærum, Denmark) and 0,1% Azide (Cat no. AMPQ52300.0500, Ampliqon, Skovlunde, Denmark)) at 2*10⁶ c/ml and kept on ice. In a 96 well round bottom plate (Cat. no. 650201, Greiner Bio-one, Frickenhausen, Germany) 50 µL/w aCD27 mAb or

Fab was added at two times the desired final concentration (the final concentration used was 10 µg/ml, 1.0 µg/ml, 0.1 µg/ml, 0.01 µg/ml, 0.001 µg/ml, 0.0001 µg/ml and 0 µg/ml. 25 µL/w cell suspension was added containing either 50.000 c/well HEK293/hCD27 cells or PBMCs. 25 µL/w rhCD70-FLAG was added (SEQ ID NO: 37) at 0.2 µg/ml final concentration. The cells incubated 1h on ice. Relevant isotype was included. 150 µL/w FACS buffer was added,

the plate was centrifuged 300 g, 5 min. The supernatant was discarded. 200 µL/w FACS buffer was added and the plate was centrifuges at 300 g, 5 min. Finally the cells were resuspended in 50 µL/w secondary Ab (anti-FLAG mAb, cat. no. AD059F, Perkin Elmer, Massachusetts, USA) diluted 1:200 in FACS buffer. The cells were incubated on ice for 30 mins. The washing procedure was repeated. Cells were resuspended in 100 µL PBS. A

dead/live marker was included in control wells in order to ensure gating on living cells (7AAD, Cat. no. 559925, BD Biosciences, NJ, USA). The cells were analysed by flow cytometry on FACS Canto (BD Biosciences, NJ, USA). Cells alone, rhCD70-FLAG alone, secondary antibody alone and relevant isotype controls were included as controls.

Antibodies and Fabs were titrated from 10 µg/ml to 0.0001 µg/ml in a 10-fold dilution row and plotted by using GraphPad Prism version 6. Curves were fitted by using a nonlinear four parameter fit. The concentration in nM that resulted in 50% inhibition of rhCD70-FLAG binding to HEK293/hCD27 cells was calculated from the curves (Table 7). Fab 2F2 was identified as the most potent inhibitor. When comparing 2F2 hlgG4 with a Fab fragment thereof, around 2-3 fold potency was lost. Four Fabs derived from the rabbit (F14, F15, F2 and F22) were identified as inhibitory, however the curve shape was not sigmoidal and they were not able to reach 100% inhibition. Hence, no IC50 value could be calculated on these four mAbs.

Table 7: IC50 values of α-hCD27 Fabs and mAbs. competition with rhCD70

Clone ID	Enzymatically cleaved Fab	Rec. hlgG4 chimeric Fab	Rec hlgG4 chimeric mAb
2F2	0.4 nM	0.2 nM	0.12 nM
5F24	Non competing		
5F32	0.9 nM		
10F13	1.4 nM		
10F31	0.7 nM		
11F26	14 nM		
Fab 151		0.7 nM	
Fab 152		0.5 nM	
Fab 153		*	
Fab 154		*	
Fab 155		*	
Fab 156		*	

*Curves did not reach 100% inhibition and were not sigmoidal, hence fitting was not possible and no IC50 value could be calculated.

Example 17: CD27 Reporter Gene Assay

CD27 (CD27_HUMAN, uniprot: P26842) is a member of the TNF receptor family and associates with TRAF2 and TRAF3 (Gravestien et. al. 1998; Yamamoto et al. 1998). Binding of CD27 ligand, CD70, induces NFκB activation in HEK293 cells (Yamamoto et al. 1998) and this knowledge was utilised to build a reporter gene assay. In transient transfections we have seen that high levels of CD27 expression induce high NFκB activation and the addition of CD70 only adds minor activity. When exchanging the high

expressing CMV promoter with a minimal promoter in the CD27 expression vector we reduced the high constitutive NFkappaB activity to a low basal activity. On the other hand when the basal activity was low the activity could be increased several fold by addition of CD70.

5 The HEK293 cell line was stably transfected with a human CD27 expression plasmid and a NFkappaB-luciferase reporter plasmid, the plasmids were transfected using Eugene transfection reagent (Roche) according to the manufacturers protocol. Hygromycin 100 microgram/ml and G418 200 microgram/ml selection agents were used for selection.

10 The CD27 expression plasmid was based on pcDNA3,1(hygro)+ (Invitrogen, Cat. No. V875-20.) and contained a hygromycin resistance gene regulated by the SV40 promoter, the CMV promoter was removed and two heat-shock-elements (HSE), a minimal c-fos promoter (SEQ ID NO: 9) and human CD27 coding sequence (refseq mRNA NM_001242.4) were inserted. Human CD27 coding sequence was PCR-amplified from the DNA IMAGE clone # 4575359 (Genbank Accession No. BC012160) using a forward and reverse primer
15 (SEQ ID NO: 16 and 17). The PCR-product was digested with BamHI and XhoI restriction enzymes and was also inserted the pcDNA3,1(hygro)+ vector. The minimal cFos promoter was amplified from human genomic DNA using forward (SEQ ID NO:22) and reverse primer (SEQ ID NO: 23). The 243 bp PCR product was digested with Bgl II and Hind III restriction enzymes and inserted in the pGL3-basic vector (Promega). The 2xHSE was made by
20 annealing two long oligonucleotides (SEQ ID NO: 24 and 25) and inserting it into the Mlu I and Bgl II restriction sites. The fusion of 2xHSE+minimal cFos promoter (seq ID NO: 9) was moved Mlu I – Hind III to pcDNA3,1(hygro)+ already containing the CD27 coding sequence.

The reporter plasmid contains three NFkappaB binding sites and a minimal interferon promoter placed in front of the coding region of the Firefly Luciferase gene. The
25 NFkappaB binding sites, the minimal interferon promoter and the coding region of the Firefly Luciferase gene (SEQ ID NO: 8) were bought as synthetic DNA (Eurofins MWG Operon, Germany) and inserted in pcDNA3,1(neomycin)- (Invitrogen, Cat. No.V795-20) using the MluI and EcoRI restriction enzymes.

A CD27/HEK293 stable reporter gene cell line, named Gudrun20, gave 10 fold
30 response in luciferase activity upon 1 microgram/ml CD70. The procedure to detect the effect of CD70 and antagonising antibodies/Fab' is was as follows. The Gudrun20 cells seeded 20.000 cells/well in polyD-lysine coated black view 96 well plates. The following day CD70 was added to the wells and after 4 hours of incubation the Steady-GLO kit (Promega) used and luminescence was monitored on a TopCount NXT (Perkin Elmer) instrument. In order to

determine a neutralising effect, antagonising antibodies or Fab's were preincubated with cells for 15 minutes before addition of CD70.

Example 18 – anti-CD27 antibodies and their Fab' derivatives tested in

5 reporter gene assay

Hybridomas of the first immunisations of mouse with human CD27 protein gave us three antibodies that bound well to the CD27 molecules, but the functional effect should also be evaluated. The 2F2, 1F16 and 1F22 and the derived Fab' fragment were tested in the human CD27 reporter cell line (Gudrun20) in the presence of 1 microgram/ml Fc-TNC-CD70 (SEQ ID NO: 27). The 2F2 mAb and Fab' had both an IC50 at 1 nM, the 1F16 mAb an IC50 of 257 nM and the Fab' of 349 nM, which also is in the same order. The 1F22 mAb has an IC50 of 1 nM like 2F2, but the 1F22 derived Fab' had an IC50 of 32 nM. Thus, the most potent Fab' in this assay is the 2F2.

15 *Table 8: Potency determined by reporter gene assay of mAbs and the derived Fab's, in presence of 1 microgram/ml CD70*

	recombinant mAb	recombinant Fab'	mAb	enzymatic cleaved Fab'
2F2	1	1		
1F16			257	349
1F22			1	32

There is a potential risk that antibodies, which by nature are bivalent, against CD27 can bind two independent receptor complexes and by bringing them together perhaps induce a signalling event. By omitting the Fc-TNC-CD70 (SEQ ID NO: 27) from the reporter gene assay, it could be tested whether the antibodies have agonistic effects. Fc-TNC-CD70 (SEQ ID NO: 27) at a concentration of 1 microgram/ml induced a 6 fold activation in the reporter gene assay.

25 *Table 9: Agonism of CD27 antibodies and the derived Fab's. Fold induction of human CD27 reporter gene assay in presence mAbs or Fab's without any CD70.*

	recombinant mAb	recombinant Fab'	mAb	enzymatic cleaved Fab'
2F2	1,5	1,2		

1F16			1,7	1,2
F22			1,3	1,1

The data shown in Table 9 show that bivalent binding by the antibodies to the CD27 receptor on induces very weak antagonism and the effect of the Fab's were even lower. Activity from 1 microgram/ml was six-fold in this assay. However, one could imagine that anti-drug-antibodies generated in patients could cross-bind the antibodies and induce higher agonistic effect whereas cross-binding of Fab' mimics a full antibody, which in our study had only minor activity.

From further mouse immunisations Fab's were generated and by using a competition assay, a few of the best were selected. There was a great difference in performance of the Fab's in the reporter gene assay. 5F24 and 10F13 Fab's were the best, but 2F2A4B2 was still superior.

Table 10: Antagonism of Fab's tested in reporter gene assay in presence of 1 microgram/ml CD70

Fab' name	IC50 nM
2F2	1
5F24	3
5F32	1536
10F13	7
11F26	373
10F31	42

Rabbits were also immunised with human CD27 protein and from single B-cells RNA was isolated and recombinant Fab's were generated.

Table 11: Potency determined by reporter gene assay of rabbit anti-human-CD27 Fab's, in presence of 1 microgram/ml CD70.

Fab' name	IC50 nM
2F2	2
Fab 0151	11
Fab 0152	7
Fab 0153	4960

Fab 0154	897
Fab 0155	244
Fab 0156	886

Two of the rabbit anti-human CD27 Fab's, Rb F1 and Rb F13, have a low IC₅₀, close to the best mouse antibodies isolated.

5 **Example 19: Effect of Anti-CD27 antibodies in B cell assay**

The effect of anti-CD27 antibodies or Fab fragments was tested in a two-cell based assay consisting of primary human B cells and HEK293/hCD70 cells as feeder cells. The effect of antibodies and Fabs was measured at day 7: Proliferation of CFSE-stained B cells was measured by flow cytometry, differentiation of B cells to plasma cells explored by the
10 expression the CD38 surface marker by flow cytometry, and hlgG was measured by AlphaLisa.

PBMCs were purified from buffy coat from Rigshospitalet. The buffy coat was transferred to a sterile flask (V=50 ml) and 100 ml RPMI 1640 + Glutamax (cat. no. 61870, Gibco Life Technologies, Nærum, Denmark) was added. 12.5 ml Ficoll-Paque Plus (Cat. no.
15 17-1440-02, GE Healthcare, Life Sciences) was added to six 50 ml tubes. Carefully, 25 ml diluted buffy coat was added on top of Ficoll-Paque by slowly pipetting down the side of the tube. The tubes were centrifuged 30 min, 950 x g, without brake. The interphase was transferred to new tube with pasteur pipettes by pooling the six tubes to three tubes. RPMI was added up to 45 ml. The tubes were centrifuged 10 min at 350 x g. The supernatant was
20 discarded and cells were resuspended in a few ml of RPMI and pooled to two tubes. RPMI was added up to 45 ml. The tubes were centrifuged 10 min at 350 x g. The supernatant was discarded and cells were resuspended in 30 ml of RPMI in one tube and counted by Cedex HiRes (Innovatis, Roche Diagnostics, Hvidovre, Denmark). Viability of HEK293/hCD27 and PBMCs was >98%. The cells were resuspended in 80 ul MACS buffer (DPBS-Ca²⁺/-Mg²⁺
25 (Cat no. 14190-169, Gibco, Life Technologies, Nærum, Denmark) with 0.5% FCS (FCS, Cat no. 10091, Gibco, Life Technologies, Nærum, Denmark) and 2 mM EDTA (cat. no. 2103-100, BioVision, California, USA)) per 1*10⁷ cells. 20 ul CD19 MicroBeads (cat. no.130-050-301, Miltenyi, Lund, Sweeden) was added per 1*10⁷ cells and cells were incubated on ice for 15 min. 2 ml MACS buffer per 10⁷ cells was added and cells were centrifuges at 300 g, 10
30 min. Cells were resuspended in 500 ul. Magnetic separation was performed using an autoMACS Separator (Miltenyi, Lund, Sweeden). The positive fraction was collected and counted by Cedex. Viability was 97-98%.

Cells were resuspended in pre-warmed PBS, 2% FCS at 20×10^6 cells/2000 μ l. CFSE (cat. no. C34554, Invitrogen, Life Technologies, Nærum, Denmark) was added at 1 μ M and cells were incubated 10 min at 37°C in the dark. Cells were washed three times at 300 g, 10 min by adding in ice cold PBS containing 1% FCS. Cells were resuspended in complete medium (RPMI 1640 + Glutamax (cat. no. 61870, Gibco, Life Technologies, Nærum, Denmark) with 10% FCS and 1% Pen/Strep (cat. no. DE17-602E, Lonza, NJ, USA).

HEK293/CD70 cells were harvested the day before, counted and irradiated before they were seeded out in U-bound 96-well plates (Cat. no. 163320, Thermo Scientific, Roskilde, Denmark) 5000 c/well in 50 μ l/ well. 50 μ l/well anti-hCD27 mAb or Fabs were added at 5, 0.5 and 0.05 μ g/ml (final conc.) in triplicates in complete medium. 50 μ l/w rhIL2 (R&D systems, cat. no. 202-IL, UK) and rhIL10 (R&D systems, cat. no. 217-IL, UK) was added at 100 ng/ml (final concentration) in complete medium. Finally, 15.000 CFSE stained CD19+ B cells were added per well in 50 μ l. Isotype controls were included as a negative control, and anti-hCD70 mAb (clone BU69, cat no. 222-820, Ancell, Minnesota, USA) as a positive control.

After seven days in culture the supernatant was harvested for IgG measurements. Human IgG was quantified in an AlphaLISA assay using biotinylated goat-anti-human IgG Fc-gamma specific antibody (cat. no. 109-066-098, Jackson ImmunoResearch, Suffolk, UK), goat-anti-human IgG (heavy + light chain) (cat. no. 109-005-088, Jackson ImmunoResearch, Suffolk, UK) in-house conjugated to AlphaLISA acceptorbeads (cat. no. 6772003, Perkin Elmer, Massachusetts, US) and streptavidin coated donorbeads (cat. no. 6760002B, Perkin Elmer, Massachusetts, US). Test samples were diluted 5 times prior to analysis. Test sample (5 μ l) was added to a 96 well half-area optiplate (cat. no. 6005560, Perkin Elmer, Massachusetts, US). 20 μ l premixed biotinylated antibody (1 μ g/ml) and acceptorbeads (10 μ g/ml) were added and incubated at room temperature for 60 min. 20 μ l streptavidin donorbeads were added (50 μ g/ml) and incubated 60 min at room temperature. Detection was done on an Envision multilabel reader (Perkin Elmer, Massachusetts, US).

The cells were washed in 200 μ l FACS buffer (PBS (-Ca²⁺/-Mg²⁺) with 2 % FCS and 0,1% Azide (Cat no. AMPQ52300.0500, Ampliqon, Skovlunde, Denmark) per well and centrifuged at 300 g, 10 min. The cells were re-suspended in FACS buffer containing anti-hCD38-APC (cat. no. 555462, BD Biosciences, NJ, USA) diluted 1:40 and incubated for 30 min on ice. Subsequently, the cells were washed with 200 μ l FACS buffer per well twice (300g, 10 min). CD38 expression and CFSE dilution was measured by flow cytometry using FACS Canto (BD Biosciences, NJ, USA).

Table 12 contains data on percent inhibition of hlgG1 production. In each experiment cells with and without rhIL10 and rhIL2 was included. When B cells were cultured with HEK293/hCD70 in the absence of rhIL10 and rhIL2 hlgG levels were low (in the range of 2-10 ng/ml) whereas in the presence of rhIL10 and rhIL2 the hlgG levels were high (in the range of 500-2000 ng/ml) with differences between donors. The percent inhibition mediated by anti-hCD27 mAbs and Fabs was calculated using these controls. Hence 100% inhibition is defined as the hlgG level of B cells incubated without rhIL10 and rhIL2, while 0% inhibition is defined as B cells incubated with rhIL10 and rhIL2. In Table 12, the % inhibition of the selected Fabs tested at 0.5 µg/ml is depicted. The 2F2 Fab was available as both hlgG4 chimera and as enzymatically cleaved Fab generated from mouse hybridoma mAb. These two formats performed equally well. The data on 2F2 included in Table 12 is on the recombinant Fab, whereas the other Fabs are enzymatically cleaved from hybridoma mAbs.

Table 12: % inhibition of hlgG secretion mediated by CD70/IL10/IL2 from B cells in the presence of 0.5 µg/ml Fab.

	2F2 (hlgG4 chimera)	5F24	5F32	10F13	10F31	11F26
Donor 1	84%	62%	67%			
Donor 2	90%	66%	67%			
Donor 3	83%	83%	82%			
Donor 4	85%			61%	52%	
Donor 5	90%			28%	30%	
Donor 6	85%			66%	36%	
Donor 7	84%	93%	89%	64%	52%	12%
Donor 8	66%	57%	56%	36%	1%	1%
Donor 9	93%					69%
Donor 10	65%					26%

2F2, 5F24 and 5F32 were potent inhibitors, while 10F13, 10F31 and 11F26 were more variable in their ability to inhibit.

When certain full mAbs were converted into Fab by cleavage, they were found to have surprisingly enhanced antagonistic effects. This was the case for 2F2, 5F24 and 5F32 Fabs, which were more potent than the corresponding mAbs. Table 12a, below, depicts % inhibition of hlgG secretion of human B cells induced by presence of CD70 stimulation (Hek293/hCD70), IL2 and IL10 in the presence of either mAbs or Fabs.

5 *Table 12a. % Inhibition of hlgG secretion mediated by CD70/IL10/IL2 from B cells in the presence of 0.5 µg/ml Fab*

	Clone ID	Enz. produced Fab	Hybridoma mAb
Donor 1	2F2	70%	31%
	5F24	56%	43%
	5F32	81%	50%
Donor 2	2F2	60%	25%
	5F24	62%	68%
	5F32	67%	27%
Donor 3	8F3	26%	36%
	8F4	54%	65%
	8F8	13%	44%

The 2F2 Fab was more than twice as potent as the mAb.

10 **Example 20: Primary human T/B cell assay with anti-CD2 and rCD70 stimulation of PBMC**

To show co-stimulatory effects of the CD27/CD70 pathway on human primary T and B cells, human PBMCs were stimulated with anti-CD2 Abs and recombinant rhCD70-Fc.

Downregulation of the CD27/CD70 co-stimulatory signal was show by inhibiting proliferative
15 T- and B cells response by anti-CD27 Fabs and mAbs.

Primary human PBMC were prepared from buffy coats (Danish blood bank) by Ficoll-Hypaque density gradient centrifugation (Histopague cat.no 10771 Sigma Aldrich) according to the protocol of the manufacturer. In short dilute anticoagulated blood has been diluted 3x in PBS and centrifugated 10 minutes at 1200 x g and RT in a swinging bucket
20 rotor. The enriched cell fraction (lymphocytes / PBMC's) was harvested, diluted to 50ml with PBS and subsequently centrifugate for 5 minutes at 400 x g at room temperature with a total of 3 washes. The red blood cells were lysed with RBC lysis buffer (RBC lysis buffer cat.no 00-4333-57 e-Bioscience) according to protocol. Finally, cells were washed twice in PBS.

Triplicates of human PBMCs were stimulated in flatbottom 96-well plates at 100.000 cells /well in 200µl with a cocktail of 3 anti-CD2 antibodies (1 µg/ml of clone CLB-T11.2/1 4B2 (cat no M1652), CLB-T11/2, 6G4 (cat no M1651) and CLB-HIK27 (cat no M1653) from Sanquin-PelliCluster, NL) and recombinant human CD70-Fc (0.1µg/ml, hFc-TNC-CD70 (SEQ ID NO: 27) and anti-hCD27 Fab 2F2 (10, 1, 0.1 and 0.01 µg/ml,) and anti-hCD27 mAb (10, 1, 0.1 and 0.01 µg/ml). Anti-CD3 OKT3 Ab (plate-bound 5 µg/ml, cat no 16-0035-85 www.eBioscience.com) was used as responsive control and human IgG4 Ab (10 µg/ml, cat no 14764, Sigma-Aldrich) as negative control.

After 3 days, PBMCs were stained with antibodies for T cells (CD4-PB (Pacific blue) BD cat. # 558116, (fx 1:100) and B cells (CD19-PE-Cy7, BD cat. # 341113 (5µl/well) and Ki67 (Ki67-PE 20 µl/well, BD cat no 556027, staining according to the manufactures protocol) and the proliferation of T- and B cells were measured by flow cytometry on a BD LSRII. Anti-CD3 stimulation was used as positive control for donor responsiveness, IgG4 isotype controls as negative controls. Proliferative responses of CD4+ gated cells and CD19+ gated cells were calculated as % of Ki67+ cells (compared to unstained controls) and CD2 +rCD70-Fc stimulation was set as 100%. Representative results are shown in Table 13.

Table 13: Proliferation of T cells in the absence or presence of 2F2 as rhlgG4 Fab.

stimuli	Proliferation T cells
aCD2	35.2
aCD2+CD70	100
+ mAb 10µg/ml	40.9
+ mAb 1µg/ml	33.0
+ mAb 0.1µg/ml	56.1
+ mAb 0.01µg/ml	86.1
+ Fab 10µg/ml	35.1
+ Fab 1µg/ml	34.5
+ Fab 0.1µg/ml	55.7
+ Fab 0.01µg/ml	83.8
Anti-CD3 (no CD2/CD70)	69.0
IgG4 control 10µg/ml	83.1

Example 21: Human CD4 memory response assay

Human CD4⁺ memory T cells can be subdivided by CD45RA and CCR7 into naïve N (CD45RA⁺ CCR7⁺), effector memory cells EM (CD45RA⁻ CCR7⁻), central memory CM (CD45RA⁻ CCR7⁺) and terminally differentiated effector cells TEMRA (CD45RA⁺ CCR7⁻).

5 Memory cells can be further divided into CD27⁺ and CD27⁻ memory cells.

To determine the effect of CD27/CD70 co-stimulation on CD4 effector memory cells, CD27⁺CCR7⁻ effector memory T cells from PBMCs (preparation see "Primary human T/B cell assay") from CMV positive donors have been purified by FACS on an ARIAII from BD and the purified subsets are stimulated with CMV lysate (1:250 cat no CMV-CM-100

10 NativeAntigen, USA) pulsed monocytes for 10 days with or without anti-CD27 Fabs and anti-CD27 mAbs. On day 5 cells are stimulated with rIL-15 (0.5 ng/ml cat no 247-IL-025, R&Dsystems) and rIL-21-Fc (0.1 ng/ml cat no 991-R2-100 R&Dsystems) . On day 10 cultures are incubated with CMV lysate (1:250), anti- CD28 (1 µg/ml cat no 304309 biolegend, USA) and anti CD49d (1µg/ml cat no 304309, biolegend USA) for 6 hs. Brefeldin A (10 µg/ml, cat
15 no B6542 Sigma-Aldrich) is added for the final 4 hs. EDTA is added for 15min at RT and cells are washed PBS. Cells are stained for CD3, CD4 and CD8 for 15min at R, washed twice with PBS and permeabilised with FACSPerm 2 (BD biosciences, USA) for 10min, washed again and stained intracellular for IFNγ and IL-2 for 30min at RT. Cells are fixed in 0.5% paraformaldehyde and analysed on a LSR II from BD.

20

Example 22: CD8-memory cell expansion assay

The CD27/CD70 co-stimulatory pathway support CD8⁺ T cell survival and expansion. CD8⁺ T cells are stimulated with CEF lysate and used to evaluate the inhibitory effects of anti-CD27 mAbs or Fabs.

25 Human CD8⁺ depleted PBMC (prepared as described in "Primary T/B cells assay") are pulsed with a CEF (2 µg/ml cytomegalovirus/EBV/influenza peptide pool, cat no CTL-CEF-002, CTL USA), co-cultures with CD8⁺CD27⁺CD45RA⁻ T cells with and without anti-CD27 Fabs or mAbs. On day 3, cultures are incubated with CEF peptides (2 µg/ml), anti-CD28 (1 µg/ml cat no 304309 biolegend, USA) and anti CD49d (1µg/ml cat no 304309,
30 biolegend USA) for 6 hs. Brefeldin A (10 µg/ml, cat no B6542 Sigma-Aldrich) is added for the final 4 hs. EDTA is added for 15min at RT and cells are washed PBS. Cells are stained for CD3, CD4 and CD8 for 15min at R, washed twice with PBS and permeabilised with FACSPerm 2 (BD biosciences, USA) for 10min, washed again and stained intracellular for IFNγ and IL-2 for 30min at RT. Cells are fixed in 0.5% paraformaldehyde and analysed
35 on a LSR II from BD.

Example 23: Mouse CD4 T cell proliferation assay

In order to determine if the ligation of CD27 with its ligand CD70 induces the proliferation of T cells, purified CD4 T cells were activated by anti-CD3 and exogenous mCD70 and proliferation was measured by Thymidine (^3H) incorporation four days later.

CD4 T cells were isolated from mouse splenocytes by negative selection using negative selection kit from Miltenyi according to the manufacturer's instructions (Miltenyi Biotech GmbH, cat.no. 130-095-248). 1.5×10^5 CD4 T cells were cultivated in RPMI 1640 + Glutamax-I medium (Invitrogen, cat.no. 61870010) containing 10% FCS (Invitrogen, cat.no. 16000-044), 1% P/S (Invitrogen, cat.no. 15140-122), 50 μM β -Mercaptoethanol (Invitrogen, cat.no. 31350-010), 1 mM Sodium Pyruvate (Invitrogen, cat.no. 11360-039) and MEM Non-Essential Amino Acids (Invitrogen, cat.no. 11140-035) and stimulated by 0.1 $\mu\text{g}/\text{ml}$ plate-bound purified hamster anti-CD3e monoclonal antibody (BD, cat.no. 553057, clone 145-2c11). Exogenous mCD70 was added to the CD4 T cell cultures in triplicates using 0, 3, 11, 33, 111, 333 and 1000 ng/ml hFc-TNC-mCD70-ECD (SEQ ID NO: 37). Three days after activation of CD4 T cells by anti-CD3e and rmCD70, 0.5 μCi ^3H -Thymidine (Amersham®, cat.no. TRK-565) was added to the cells per well and proliferation was measured by Thymidine incorporation 16 hours later.

As shown in Table 14, the numbers of proliferating ^3H -cells measured by Topcount demonstrated that the anti-CD3 induced CD4 T cell proliferation could be enhanced by adding increasing amounts of rmCD70 in a dose dependent manner. This indicated that CD27 acts as a co-stimulatory molecule on CD4 T cells, supporting their proliferation in an even more pronounced manner than was gained by adding 0.5 $\mu\text{g}/\text{ml}$ of the agonising anti-CD28 monoclonal antibody (BD, cat.no. 553294, clone 37.51).

Table 14: Numbers of ^3H -cells measured in Topcount on day 4 after stimulation.

stimulated with 0.1 $\mu\text{g}/\text{ml}$ p.b. anti-CD3

	well 1	well 2	well 3	mean value
+1000 ng/ml CD70	223976	214184	208335	215498.3333
+333 ng/ml CD70	219249	208791	210528	212856
+111 ng/ml CD70	213229	208435	209055	210239.6667
+33 ng/ml CD70	203605	203354	200237	202398.6667
+11 ng/ml CD70	177666	187825	177993	181161.3333
+3 ng/ml CD70	176801	162091	159449	166113.6667
None	159190	171208	163633	164677

+ 500 ng/ml anti-CD28		182278	191068	189734		187693.3333
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Example 24: Mouse CD4 T and B cell co-culture assay

In order to determine if the ligation of CD27 with its ligand CD70 induces the activation or differentiation of T and B cells, respectively, T and B cell co-cultures were activated by anti-CD3 and exogenous mCD70. 4 days later, the expression of activation/differentiation markers were analyzed on the cell surface by flow cytometry analysis (FACS).

CD4 T and naïve B cells were isolated from mouse splenocytes by negative selection using negative selection kit from Miltenyi according to the manufacturer's instructions (Miltenyi Biotech GmbH, cat.no. 130-095-248 and 130-090-862). 5×10^5 CD4 T and 5×10^4 cells were cultivated in RPMI 1640 + Glutamax-I medium (Invitrogen, cat.no. 61870010) containing 10% FCS (Invitrogen, cat.no. 16000-044), 1% P/S (Invitrogen, cat.no. 15140-122), 50 μ M β -Mercaptoethanol (Invitrogen, cat.no. 31350-010), 1 mM Sodium Pyruvate (Invitrogen, cat.no. 11360-039) and MEM Non-Essential Amino Acids (Invitrogen, cat.no. 11140-035) and stimulated by 0.1 μ g/ml plate-bound purified hamster anti-CD3e monoclonal antibody (BD, cat.no. 553057, clone 145-2c11). Exogenous mCD70 was added to the T and B cell co-cultures in triplicates using 0, 3, 11, 33, 111, 333 and 1000 ng/ml hFc-TNC-mCD70-ECD (SEQ ID NO: 37). Four days after stimulation of the cells by anti-CD3e and rmCD70, FACS analysis of the cell surface markers CD4, CD19, CD38, CD25 and IgM was performed by using anti-CD4 Qdot605 (Invitrogen, cat.no. Q10092, 1:200 dilution), anti-CD19 AF700 (eBioscience, cat.no. 56-0193, 1:200 dilution), anti-CD38 FITC (BD, cat.no. 558813, 1:100 dilution), anti-CD25 PerCP-Cy5.5 (BD, cat.no. 551071, 1:200 dilution) and anti-IgM APC (Jackson Immuno Research, cat.no. 115-006-020, 1:200 dilution) for extracellular cell stain.

In Tables 15-19, the percentages (%) and median fluorescent intensities (MFI) of the above mentioned cell surface markers, expressed four days after stimulation, are listed. The data indicate that the addition of rmCD70 had an impact on CD4+ T and CD19+ B cell activation/ differentiation as well on their frequency. The frequency of both CD4+ T (Table 15) and CD19+ B cells (Table 16) was increased. The activation marker CD25 was up-regulated on CD4 T cells and reached its maximal expression at a rmCD70 concentration of 33 and 111 ng/ml (Table 17). Under these conditions, the surface IgM expression was down-regulated on CD19+ B cells (Table 19), comparable to the expression on cells that were co-stimulated with 0.5 μ g/ml of the agonising anti-CD28 monoclonal antibody (BD, cat.no. 553294, clone 37.51).

However, CD38⁺ CD19⁺ B cells increase was dependent on exogenous rmCD70, in a dose dependent manner (Table 18). These findings indicated that CD27 acts as a co-stimulatory molecule on CD4 T cells and supported the activation/differentiation of B cells.

5 *Table 15: Frequency of CD4⁺ T cells shown in % of living cells, analyzed by FACS on day 4 after stimulation.*

stimulated with 0.1 µg/ml p.b. anti CD3

	well 1	well 2	well 3	mean value
+1000 ng/ml CD70	52.2	55.5	52.5	53.4
+333 ng/ml CD70	54.9	57.5	54.8	55.7
+111 ng/ml CD70	54.4	56.3	54.4	55
+33 ng/ml CD70	55	59.6	57.4	57.3
+11 ng/ml CD70	49.3	51	53.4	51.2
+3 ng/ml CD70	51.7	49.3	47.2	49.4
None	49.1	48.6	47.6	48.4
+ 5 µg/ml anti-CD28	66.3	65.1		65.7

Table 16: Frequency of CD19⁺ B cells shown in % of living cells, analyzed by FACS on day 4 after stimulation.

stimulated with 0.1 µg/ml p.b. anti-CD3

	well 1	well 2	well 3	mean value
+1000 ng/ml CD70	10.9	10.2	10.5	10.53
+333 ng/ml CD70	9.1	9.2	8.9	9.07
+111 ng/ml CD70	8.9	8.2	9.4	8.83
+33 ng/ml CD70	7	6.7	7.3	7.00
+11 ng/ml CD70	6	5.8	6.5	6.10
+3 ng/ml CD70	6	5.7	6.3	6.00
None	5.7	6.2	6.9	6.27
+ 5 µg/ml anti-CD28	5.6	5.4		5.50

Table 17: Frequency of CD25+ CD4 T cells shown in % of living cells, analyzed by FACS on day 4 after stimulation.

stimulated with 0.1 µg/ml p.b. anti-CD3

	well 1	well 2	well 3	mean value
+1000 ng/ml CD70	54.4	44.5	45.7	48.20
+333 ng/ml CD70	57.2	55	59.5	57.23
+111 ng/ml CD70	63.5	60	63.7	62.40
+33 ng/ml CD70	64.1	65.9	65.3	65.10
+11 ng/ml CD70	44.5	52.1	47.7	48.10
+3 ng/ml CD70	42.6	39.3	39.2	40.37
None	28.4	31.7	28.3	29.47
+ 5 µg/ml anti-CD28	76.2	76.3		29.47

Table 18: Frequency of CD38+ CD19 B cells shown in % of living cells, analyzed by FACS on day 4 after stimulation.

5

stimulated with 0.1 µg/ml p.b. anti-CD3

	well 1	well 2	well 3	mean value
+1000 ng/ml CD70	9.5	8.1	8.3	8.63
+333 ng/ml CD70	7.7	7.4	6.9	7.33
+111 ng/ml CD70	7.5	7.1	7.3	7.30
+33 ng/ml CD70	6.4	6	6.9	6.43
+11 ng/ml CD70	5.3	5.2	5.8	5.43
+3 ng/ml CD70	5.6	5.1	5.3	5.33
None	4.8	5.3	5.9	5.33
+ 5 µg/ml anti-CD28	4.2	4		5.33

Table 19: Mean fluorescence intensity (MFI) of IgM on CD19 B cells shown, analyzed by FACS on day 4 after stimulation.

stimulated with 0.1 µg/ml p.b. anti-CD3

	well 1	well 2	well 3	mean value
+1000 ng/ml CD70	6173	7232	7094	6833.00
+333 ng/ml CD70	5438	5218	5317	6833.00
+111 ng/ml CD70	4553	4315	4850	5324.33
+33 ng/ml CD70	4510	5185	5021	4572.67
+11 ng/ml CD70	7384	7020	6372	4905.33
+3 ng/ml CD70	7100	8137	7325	6925.33
None	8465	7902	7783	7520.67
+ 5 µg/ml anti-CD28	4434	3058	3276	7520.67

Example 25: Cross-linking of anti-CD27 mAbs and Fabs with a secondary

5 antibody

In order to determine the potential agonistic effect of cross-linking anti-CD27 mAb or Fab with a secondary antibody, an assay is performed where purified CD4⁺ T cells from healthy human donors are stimulated with PMA in the presence of anti-CD27 mAb or Fab and +/- cross-linking secondary antibody.

- 10 CD4⁺ T cells are purified from buffy coats from healthy donors according to the manufacturer's specification (Human CD4⁺ T Cell Enrichment cocktail, Stemcell Technologies, cat # 15062). After purification, remaining red blood cells are removed by incubation of cells in 5 ml of Red Blood Cell (RBC) lysis buffer (eBioscience, cat # 00-4333-57) for 5 min at room temperature before washing the samples twice in PBS (Gibco, cat #
- 15 10010), centrifuge for 5 min at 400g between washes. Purified CD4⁺ T cells are resuspended in RPMI 1640 + Glutamax medium (Gibco cat# 61870-010) supplemented with 10% FCS (Gibco cat# 16140-071) and 100U/ml penicillin/100µg/ml streptomycin (Gibco Cat# 15140130) and seeded in round bottom 96 wells plates (BD cat# 353077) at 1x10⁵ cells/well. Cells are cultured in 1 ng/ml PMA (Calbiochem, cat # 524400) with different concentrations
- 20 of anti-CD27 (2F2A4B2 mAb or Fab) with or without a secondary antibody in a final volume of 0.2ml RPMI 1640 medium per well. Cells are incubated for 48 hrs at 37°C in a humidified atmosphere with 5% CO₂, before pulsed with 0.5 µCi of [³H]thymidine/well (PerkinElmer).

After an additional 18 hrs of incubation, the cells are harvested, and [³H]thymidine incorporation is measured by a beta counter (Topcount NXT, PerkinElmer).

Example 26: Humanization of anti-hCD27 antibody 5F24

5 The humanized forms of antibody have variable framework region(s) substantially from a human immunoglobulin (termed an acceptor immunoglobulin) and CDRs substantially from a non-human Mab 5F24. Back mutations which might be critical for maintaining the binding affinity and specificity were predicted by 3D model analysis. All back mutations are marked with bold fonts in 0307-0000-0197. The humanized forms of antibody with or without
10 back mutations were produced and evaluated by SPR and reporter gene assay.

Experimental data demonstrated that back mutations on both light chain and heavy chain were important for maintaining affinity and potency of humanized antibody. In order to evaluate the impact of individual back mutations and eliminate the unnecessary ones from humanized antibody, the specific mutated residue in the humanized antibody bearing full set
15 of back mutations was replaced by its germline counterpart, respectively.

Table 20: Protein sequences of 5F24-derived Fab fragments:

SEQ ID NO	Clone	Heavy chain sequence	Light chain sequence
60	0307-0000-0185	QVQLQQPGAELVRPGTSVRLSCKA SNYSFTNFWMNWVRQRPQGGL WIGMIHPDSETRLNQQFKDKATLT VDKSSSTAYMQLSSPTSEDSAVYY CARLDNDYDALDYWGQGTSVTVS SASTKGPSVFPLAPCSRSTSESTAA LGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVTVPS SSLGTKTYTCNVDHKPSNTKVDKR VESKYGPP	DIQMNQSPSSLSASLGDTITITCH ASQNFNVWLSWYQQKPGNIPQL LIYKASNLHTGVPSRFSGSGSGT DFTLTISLQPEDIGTYCYCQQGQS YPWTFGGGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSLSSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF NRGEC
61	0307-0000-0186	QVQLVQSGAEVKKPGASVKVSKA SGYTFTNFWMNWVRQAPQGGL WMGMIHPDSETRYAQKFQGRVT MTRDTSTSTVYMELSSLRSEDYAV	DIQMTQSPSSLSASVGDRTITITCH ASQNFNVWLSWYQQKPGKAPKL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDIATYCYCQQGQS

		YYCARLDNDYDALDYWGQGTMT VSSASTKGPSVFPLAPCSRSTSEST AALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVT PSSSLGKTYTCNVDHKPSNTKVD KRVESKYGPP	YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSTYLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF NRGEC
62	0307- 0000- 0189	QVQLQQSGAEVKKPGASVKVSCK ASGYSFTNFWMNWVRQAPGQGLE WMGMIHPDSETRLNQKFQGRVT TVDKSTSTAYMELSSLRSEDATVY CARLDNDYDALDYWGQGTMTVS SASTKGPSVFPLAPCSRSTSESTAA LGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVP SSSLGKTYTCNVDHKPSNTKVDKR VESKYGPP	DIQMTQSPSSLSASVGDRVITICH ASQNFNVWLSWYQQKPGKAPKL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDATYYCQQGQS YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSTYLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF NRGEC
63	0307- 0000- 0197	QVQLQQSGAEVKKPGASVKVSCK ASGYSFTNFWMNWVRQAPGQGLE WMGMIHPDSETRLNQKFQGRVT LTVDKSTSTAYMELSSLRSEDATV YYCARLDNDYDALDYWGQGTMT VSSASTKGPSVFPLAPCSRSTSEST AALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVT PSSSLGKTYTCNVDHKPSNTKVD KRVESKYGPP	DIQMTQSPSSLSASVGDRVITICH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDATYYCQQGQS YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSTYLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF NRGEC
64	0307- 0000- 0198	QVQLVQSGAEVKKPGASVKVSCKA SGYTFTNFWMNWVRQAPGQGLE WMGMIHPDSETRYAQKFQGRVT MTRDTSTSTVYMELSSLRSEDATV YYCARLDNDYDALDYWGQGTMT VSSASTKGPSVFPLAPCSRSTSEST AALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVT PSSSLGKTYTCNVDHKPSNTKVD	DIQMTQSPSSLSASVGDRVITICH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDATYYCQQGQS YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSTYLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF

		KRVESKYGPP	NRGEC
65	0307-0000-0228	QVQLVQSGAEVKKPGASVKVSCKA SGYSFTNFWMNWVRQAPGQGLE WMGMIHPDSETRLNQKFQGRVTL TVDKSTSTAYMELSSLRSEDVAVYY CARLDNDYDALDYWGQGTMTVVS SASTKGPSVFPLAPCSRSTSESTAA LGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVTVPS SSLGKTYTCNVDHKPSNTKVDKR VESKYGPP	DIQMTQSPSSLSASVGDRVITICH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDATYYCQQGQS YPWTFGQGTKEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSLSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF NRGEC
66	0307-0000-0229	QVQLQQSGAEVKKPGASV KVSCKASGYFTNFWMNWVRQAP GQGLEWMGMIHPDSETRLNQKF QGRVTLTVDKSTSTAYMELSSLRS EDTAVYYCARLDNDYDALDYWGQ GTMVTVSSASTKGPSVFPLAPCSR STSESTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYS LSSVTVPSSSLGKTYTCNVDHKPS NTKVDKRVESKYGPP	DIQMTQSPSSLSASVGD RVTITCHASQNFNVWLSWYQQK PGKAPQLLIYKASNLHTGVPSRFS GSGSGTDFTFTISLQPEDATYY CQQGQSYPWTFGQGTKEIKRT VAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSTLT LSKADYEKHKVYACEVTHQGLS SPVTKSFNRGEC
67	0307-0000-0230	QVQLQQSGAEVKKPGASVKVSCK ASGYFTNFWMNWVRQAPGQGLE WMGMIHPDSETRYNQKFQGRVT LTVDKSTSTAYMELSSLRSEDVAVY YCARLDNDYDALDYWGQGTMTV SSASTKGPSVFPLAPCSRSTSESTA ALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVTVPS SSLGKTYTCNVDHKPSNTKVDK RVESKYGPP	DIQMTQSPSSLSASVGD RVTITCHASQNFNVWLSWYQQK PGKAPQLLIYKASNLHTGVPSRFS GSGSGTDFTFTISLQPEDATYY CQQGQSYPWTFGQGTKEIKRT VAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSTLT LSKADYEKHKVYACEVTHQGLS SPVTKSFNRGEC
68	0307-0000-0231	QVQLQQSGAEVKKPGASV KVSCKASGYFTNFWMNWVRQAP GQGLEWMGMIHPDSETRLAQKF QGRVTLTVDKSTSTAYMELSSLRS	DIQMTQSPSSLSASVGD RVTITCHASQNFNVWLSWYQQK PGKAPQLLIYKASNLHTGVPSRFS GSGSGTDFTFTISLQPEDATYY

		EDTAVYYCARLDNDYDALDYWGQ GTMVTVSSASTKGPSVFPLAPCSR STSESTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGKTYTCNVDHKP SNTKVDKRVESKYGPP	CQQGQSYPWTFGQGTKVEIKRT VAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSTL TLKADYEKHKVYACEVTHQGLS SPVTKSFNRGEC
69	0307- 0000- 0232	QVQLQQSGAEVKKPGASVKVSCK ASGYSFTNFWMNWVRQAPGQGLE WMGMIHPDSETRLNQKFQGRVT MTVDKSTSTAYMELSSLRSEDYAV YYCARLDNDYDALDYWGQGTMTV VSSASTKGPSVFPLAPCSRSTSEST AALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTV PSSSLGKTYTCNVDHKPSNTKVD KRVESKYGPP	DIQMTQSPSSLSASVGDRVTITCH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDYATYYCQQGQS YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSLSTLTLKADY EYKHKVYACEVTHQGLSSPVTKSF NRGEC
70	0307- 0000- 0233	QVQLQQSGAEVKKPGASVKVSCK ASGYSFTNFWMNWVRQAPGQGLE WMGMIHPDSETRLNQKFQGRVTL TRDKSTSTAYMELSSLRSEDYAVY YCARLDNDYDALDYWGQGTMTVTV SSASTKGPSVFPLAPCSRSTSESTA ALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVP SSSLGKTYTCNVDHKPSNTKVDK RVESKYGPP	DIQMTQSPSSLSASVGDRVTITCH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDYATYYCQQGQS YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSLSTLTLKADY EYKHKVYACEVTHQGLSSPVTKSF NRGEC
71	0307- 0000- 0234	QVQLQQSGAEVKKPGASVKVSCK ASGYSFTNFWMNWVRQAPGQGLE WMGMIHPDSETRLNQKFQGRVTL TVDTSTSTAYMELSSLRSEDYAVYY CARLDNDYDALDYWGQGTMTVVS SASTKGPSVFPLAPCSRSTSESTAA LGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPS SSSLGKTYTCNVDHKPSNTKVDKR	DIQMTQSPSSLSASVGDRVTITCH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDYATYYCQQGQS YPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKDYSLSTLTLKADY EYKHKVYACEVTHQGLSSPVTKSF

		VESKYGPP	NRGEC
72	0307- 0000- 0235	QVQLQQSGAEVKKPGASVKVSCK ASGYSFTNFWMNWVRQAPGQGLE WMGMIHPSDSETRLNQKFQGRVTL TVDKSTSTVYMELSSLRSED TAVYY CARLDNDYDALDYWGQGTMTVTS SASTKGPSVFPLAPCSRSTSESTAA LGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVTVPS SSLGTKTYTCNVDHKPSNTKVDKR VESKYGPP	DIQMTQSPSSLSASVGDRVITICH ASQNFNVWLSWYQQKPGKAPQL LIYKASNLHTGVPSRFSGSGSGT DFTFTISLQPEDATYYCQQGQS YPWTFGQGTKEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFY PREAKVQWKVDNALQSGNSQES VTEQDSKSTYSLSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSF NRGEC

Generation of expression vectors for 5F24-derived Fab fragments

All plasmids for expressing Fab fragments were constructed by GENEWIZ, Inc. The coding sequences for light chain or heavy chain were inserted into pJSV002, a CMV promoter-based expression vectors between EcoRI and BamHI sites. A signal peptide (mplllllpllwagala) was added before coding sequences to facilitate secretion.

Fab fragments expression

Plasmid DNA encoding the respective light chain and heavy chain were transfected with 293fectin™ reagent into Freestyle™ 293-F cells at 1:1 ratio. For protein production, cells were grown in serum-free FreeStyle 293 medium containing 4 mM glutamine, 1% PLURONIC® F68 and penicillin-streptomycin antibiotics at 1×10^6 cells per ml and incubated with shaking for 5 days at 37 °C, 8% CO₂. Supernatants were collected on day 5 post transfection by centrifugation

DNA Transfection:

The cell density of cultures used for transfection was $0.9-2.0 \times 10^6$ cells/ml.

A mix of 0.5 µg LC vector DNA + 0.5 µg HC vector DNA was used per ml cell

culture.

The DNA was diluted in Opti-MEM media (Gibco) 30µl media/µg DNA, mixed and incubated at room temperature (23-25 °C) for 5 min.

293Fectin™ (Invitrogen) was used as transfection reagent at a concentration of 1µl per µg DNA.

The 293Fectin™ was diluted 30X in Opti-MEM media (Gibco), mixed and incubated at room temperature (23-25 °C) for 5 min.

The DNA and 293Fectin solutions were mixed and left to incubate at room temperature (23-25 °C) for 25 min.

5 The DNA-293Fectin mix was then added directly to the cell culture.

The transfected cell culture was transferred to a shaker incubator at 37°C, 8 % CO₂ and 125 rpm.

5 days post transfection, cell culture supernatants were harvested by centrifugation, followed by filtration through a 0.22 µm PES filter (Corning).

10 Quantitative analysis of antibody production was performed by Biolayer Interferometry directly on clarified cell culture supernatants using the FortéBio Octet system and protein A biosensors.

Purification of 5F24-derived Fab fragments

15 Culture supernatant containing secreted Fab was harvested by centrifugation (15,000 rpm×20 min, 4°C) and then cleared by the filtration with 0.22 µm cellulose nitrate membrane. The cleared supernatant was applied to a Tricorn KappaSelect (8ml) column (GE healthcare), followed by a 5 column volume wash with PBS plus 0.5M NaCl. The bound Fab was then eluted with 0.1M Glycine pH2.7 and collected as 2ml fractions into the glass tube
20 with 200ml 1M Tris.Cl pH9.0. The eluted proteins were pooled, diluted to 100ml in 5mM Sodium Citrate, pH5.0 and loaded on a Mono S 10/100 GL(8ml) column (GE healthcare) to remove light chain dimer, followed by a 5 column volume wash with 5mM Sodium Citrate pH5.0. The protein was then eluted with a two segment linear gradient of 20mM Sodium Citrate, 1M NaCl pH5.0: 0~20% in 20 column volume followed by 20~100% in 5 column
25 volume. The peak fractions were pooled and buffer-exchanged to Phosphate Buffered Saline (PBS) with Amicon ultra 15 centrifugal units (3,000kD MWCO, Millipore). After concentrating, the final protein concentrations were determined by measuring 280nm absorbance with a NANODROP UV spectrometer. Protein purity was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Endotoxin levels were evaluated by LAL
30 test (Charles River).

SEC-HPLC and SEC-MALS analysis

Typically 50 µg protein for SEC-HPLC only or 100 µg protein for SEC-MALS was analysed on a TSKSWxl 2000 column (TOSOH) using a 1200 HPLC system (Agilent
35 Technologies). The buffer was 2×PBS, 5% isopropanol pH6.8 and flow rate was 0.5 ml/min

unless stated otherwise. The chromatograms were monitored at 280 nm and the light scattering was monitored with a miniDAWN TREOS (Wyatt). SEC-MALS data was further analysed with ASTRA software version 5.3.4 (Wyatt).

5 Measurement of binding kinetics for humanized anti-CD27 Fab fragments by SPR

In order to select high affinity binders and rank the different humanized Fab fragments, the binding constants of Fab binding to human CD27-Fc fusion protein was measured by surface plasmon resonance (SPR).

10 The experiments were performed on a BIAcore T200 instrument (GE Healthcare) and analysed with the BIAcore T200 Evaluation Software. Human CD27 (1-184)-mFc (mIgG2a) (SEQ ID NO 73):

TPAPKSCPERHYWAQGKLCCQMCEPGTFLVKDCDQHRKAAQCDPCIPGVSFSPDHHTRP
 HCESCRHCNSGLLVRNCTITANAECACRNGWQCRDKECTECDPLPNPSLTARSSQALSPH
 15 PQPTHLPYVSEMLEARTAGHMQTLADFRQLPARTLSTHWPPQRSLEPRGPTIKPCPPCKCP
 APNLLGGPSVFIFPPKIKDVLMLISLPIVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHR
 EDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPE
 EEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSGYSYFMYSKLRVEKKN
 WVERNSYSCSVVHEGLHNHHTTKSFSRTPGK) was directly immobilized at 500 RU on flow
 20 cells of a CM5 sensor chip (GE Healthcare; cat.# BR-1000-12) via amine coupling.
 Humanized Fab fragments were diluted in HBS-EP buffer with a dilution factor 3x ranging 0.6-33.3 nM. Fab fragments were injected for 180 s, followed by a 500 s dissociation phase. Binding curves were measured at 25 °C with a flow rate of 30 µL/min. Regeneration of the chip surface was performed with 1M formic acid for 15 s at 50 µl/min. Determination of the
 25 kinetic parameters was performed by fitting with a 1:1 binding model. Kinetic parameters were calculated and are stated in Table 21.

All of the 5F24-derived Fabs showed very good binding to human CD27 (1-184)-Fc. The kinetic values are for purposes only to complement the ranking of these Fabs done by other assays.

Table 21: Binding kinetics of Fab fragments to human CD27(1-184)-Fc (SEQ ID NO: 73) were determined by SPR. Kinetic parameters are shown.

	NNCD	Name	Ka (1/Ms)	Kd (1/s)	KD (M)
1 st round 5F24	0307-00000185	HZ5F24_WT	1.56E+05	8.78E-05	5.64E-10
	0307-00000186	HZ5F24_HZ	5.01E+05	2.23E-04	4.44E-10
	0307-00000189	HZ5F24_HCBM+LC	2.52E+05	4.79E-05	1.90E-10
	0307-00000197	HZ5F24_BM	2.48E+05	1.58E-05	6.36E-11
	0307-00000198	HZ5F24_HC+LCBM	2.58E+05	5.16E-05	2.00E-10
2 nd round 5F24	0307-00000228	HZ5F24_BM(HC_V5V)	3.01E+05	1.05E-04	3.50E-10
	0307-00000229	HZ5F24_BM(HC_T28T)	3.73E+05	6.79E-05	1.82E-10
	0307-00000230	HZ5F24_BM(HC_Y60Y)	1.64E+05	5.90E-05	3.59E-10
	0307-00000231	HZ5F24_BM(HC_A61A)	3.14E+05	4.06E-05	1.29E-10
	0307-00000232	HZ5F24_BM(HC_M70M)	2.31E+05	1.22E-04	5.28E-10
	0307-00000233	HZ5F24_BM(HC_R72R)	1.42E+05	1.06E-04	7.51E-10
	0307-00000234	HZ5F24_BM(HC_T74T)	1.65E+05	7.73E-05	4.68E-10
	0307-00000235	HZ5F24_BM(HC_V79V)	2.16E+05	6.05E-05	2.80E-10

CD27 Reporter Gene Assay

- 5 CD27 (CD27_HUMAN, uniprot: P26842) is a member of the TNF receptor family and associates with TRAF2 and TRAF3 (Gravestien et. al. 1998; Yamamoto et al. 1998). Binding of CD27 ligand, CD70, induces NFkappaB activation in HEK293 cells (Yamamoto et al. 1998) and this knowledge was utilised to build a reporter gene assay. In transient transfections we have seen that high levels of CD27 expression induce high NFkappaB
- 10 activation and the addition of CD70 only adds minor activity. When exchanging the high expressing CMV promoter with a minimal promoter in the CD27 expression vector we reduced the high constitutive NFkappaB activity to a low basal activity. On the other hand when the basal activity was low the activity could be increased several fold by addition of CD70.

The HEK293 cell line was stably transfected with a human CD27 expression plasmid and a NFkappaB-luciferase reporter plasmid, the plasmids were transfected using Fugene transfection reagent (Roche) according to the manufacturers protocol. Hygromycin 100 microgram/ml and G418 200 microgram/ml selection agents were used for selection.

5 The CD27 expression plasmid was based on pcDNA3,1(hygro)+ (Invitrogen, Cat. No. V875-20.) and contained a hygromycin resistance gene regulated by the SV40 promoter, the CMV promoter was removed and two heat-shock-elements (HSE), a minimal c-fos promoter (SEQ ID NO: 9) and human CD27 coding sequence (refseq mRNA NM_001242.4) were inserted. Human CD27 coding sequence was PCR-amplified from the DNA IMAGE
10 clone # 4575359 (Genbank Accession No. BC012160) using a forward and reverse primer (SEQ ID NO: 16 and 17). The PCR-product was digested with BamHI and XhoI restriction enzymes and was also inserted the pcDNA3,1(hygro)+ vector. The minimal cFos promoter was amplified from human genomic DNA using forward (SEQ ID NO:22) and reverse primer (SEQ ID NO: 23). The 243 bp PCR product was digested with Bgl II and Hind III restriction
15 enzymes and inserted in the pGL3-basic vector (Promega). The 2xHSE was made by annealing two long oligonucleotides (SEQ ID NO: 24 and 25) and inserting it into the Mlu I and Bgl II restriction sites. The fusion of 2xHSE+minimal cFos promoter (seq ID NO: 9) was moved Mlu I – Hind III to pcDNA3,1(hygro)+ already containing the CD27 coding sequence.

The reporter plasmid contains three NFkappaB binding sites and a minimal
20 interferon promoter placed in front of the coding region of the Firefly Luciferase gene. The NFkappaB binding sites, the minimal interferon promoter and the coding region of the Firefly Luciferase gene (SEQ ID NO: 8) were bought as synthetic DNA (Eurofins MWG Operon, Germany) and inserted in pcDNA3,1(neomycin)- (Invitrogen, Cat. No.V795-20) using the MluI and EcoRI restriction enzymes.

25 A CD27/HEK293 stable reporter gene cell line, named Gudrun20, gave 3-4 fold response in luciferase activity upon CD70 stimulation. The procedure to detect the effect of CD70 and antagonising antibodies/Fab' was as follows. The Gudrun20 cells were seeded 60,000 cells/well in polyD-lysine coated black view 96 well plates. The following day CD70-expressing HEK293 cells were added 10,000 cells/well to the wells. After 4 hours of
30 incubation the Steady-GLO kit (Promega) used and luminescence was monitored on a PHERAstar Plus (BMG LABTECH) instrument. In order to determine a neutralising effect, antagonising humanized 5F24 Fab's with serial dilution starting from 10µg/ml were preincubated with cells for 15 minutes before addition of CD70-expressing HEK293 cells.

SPR and RG assays demonstrated that none of the heavy chain back mutations are critical for restoring affinity and potency of humanized 5F24. In contrast, the single back mutations in light chain slightly improved potency of humanized 5F24. Therefore, molecule 0307-0000-0198, humanized 5F24 bearing single back mutation in light chain, was selected for further characterization.

Example 27: Anti-CD27 Humanization

The sequence of 5F24 was obtained from cloning of the hybridoma 5F24A1.

All numbering used in this example refers to the Kabat numbering scheme.

>5F24VH (CDRs marked with bold)

QVQLQQPGAELVRPGTSVRLSCKASNYSFT**NFWMN**WVRQRPQGQGLEWIG**MIHP****SD****SETR**
LNQKFKDKATLTVDKSSSTAYMQLSSPTSEDSAVYYCAR**LDNDYDALDY**WGQGTSTVTVSS

>5F24VL (CDRs marked with bold)

DIQMNQSPSSLSASLGDTITIT**CHASQNFNVWLS**WYQQKPGNIPQLLIY**KASNLHT**GVPS
RFGSGSGTDFLTITSLQPEDIGTYYC**QQGQSYPWT**FGGGTKVEIK

From an analysis of the 5F24 sequences, the CDRs according to Kabats definition are:

>CDR_H1

NFWMN

>CDR_H2

MIHPSDSETRLN**QKFKD**

>CDR_H3

LDNDYDALDY

>CDR_L1

HASQNFNVWLS

>CDR_L2

KASNLHT

>CDR_L3

QQGQSYPWT

A 3D model of 5F24 was built using standard techniques in MOE and all residues within 4.5 Å of the effective CDR regions (VH: 31-35B, 50-58, 95-102; VL: 24-34, 50-56, 89-97) are defined as Mask residues. Mask residues are all potentially important for sustaining the binding in the CDRs.

The mask residues include positions 1-3,5,27-37,47,49-60,69-71,73,78,91-104 for the heavy chain and 1-5,22-36,45-60,62,64-71,87-98 for the light chain.

Using germline searches and manual inspection VH1_46 and JH3 were identified as an appropriate human germline combination for the heavy chain and VKI_O18 and JK1 were identified as the appropriate human germline combination for the light chain, but other germlines are also suitable.

>VH1_46/JH3

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYMHWRQAPGQGLEWMGIINPSGGSTS
YAQKFQGRVTMTRDTSTSTVYMESSLRSEDVAVYYCAR/AFDIWGQGTMTVTVSS

>VKI_O18/JK1

DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPS
RFGSGSGTDFTFITISLQPEDATYYCQQYDNLP/WTFGQGTKVEIK

The humanization can now be performed with the following rules:

- Residues outside the mask are taken as human.
- Residues inside the mask and inside the Kabat CDR are taken as murine.
- Residues inside the mask and outside the Kabat CDR with mouse/germline consensus are taken as the consensus sequence.
- Residues inside the mask and outside the Kabat CDR with mouse/germline difference are subject to potential back mutations.

Grafting the effective CDR regions of 5F24 into the germlines forms the basic humanization construct of 5F24, hz5F24.

>hz5F24VHQVQLVQSGAEVKKPGASVKVSCKASGYTFT**NFWMN**WVRQAPGQGLEWMGM
IHPDSETRYAQKFQGRVTMTRDTSTSTVYMESSLRSEDVAVYYCARLDNDYDALDYWG
QGTMTVTVSS

>hz5F24VLDIQMTQSPSSLSASVGDRVTIT**CHASQNFNVWLS**WYQQKPGKAPKLLIY**KASN**
LHTGVPSRFGSGSGTDFTFITISLQPEDATYYCQQGQSYPWTFGQGTKVEIK

From an analysis of the hz5F24 sequences, the CDRs according to Kabats definition are:

>CDR_H1

NFWMN

>CDR_H2

MIHPDSETRYAQKFQG

>CDR_H3

LDNDYDALDY

>CDR_L1

HASQNFNVWLS

>CDR_L2

KASNLHT

5 >CDR_L3

QQGQSYPT

Any discrepancy between 5F24 and hz5F24 in a mask residue will create a potential backmutation and the list includes

hz5F24VH: V5Q, T28S, Y59L, A60N, M69L, R71V, T73K, V78A

10 hz5F24VL: K45Q

The final variable domain (hz5F24VH+V5Q,hz5F24VL+K45Q) was selected within one or more of these mutations.

Example 28: Ligand competition assay

15 Anti-hCD27 mAbs and Fabs that bind cellular hCD27 were tested for the ability to compete with binding of FLAG-tagged recombinant human (rh)-CD70 to HEK293/hCD27 cells, where binding of CD70-FLAG was monitored by a secondary anti-FLAG mAb.

HEK293 cells were detached by washing with PBS and adding Versene (Cat. no. 15040033, Gibco, Life Technologies, Nærum, Denmark) and then incubated at 37°C for 10 min. The cells were centrifuged at 300 g, 10 min and resuspended in FACS buffer (Dulbecco's Phosphate-Buffered Saline (DPBS-Ca²⁺/Mg²⁺, Cat no. 14190-169, Gibco, Life Technologies, Nærum, Denmark) with 2 % Fetal Calf Serum (FCS, Cat no. 10091, Gibco, Life Technologies, Nærum, Denmark) and 0,1% Azide (Cat no. AMPQ52300.0500, Ampliqon, Skovlunde, Denmark)) at 2*10⁶ c/ml and kept on ice. In a 96 well round bottom plate (Cat. no. 650201, Greiner Bio-one, Frickenhausen, Germany) 50 µL/w aCD27 mAb or Fab was added at two times the desired final concentration (the final concentration used was 10 µg/ml, 1.0 µg/ml, 0.1 µg/ml, 0.01 µg/ml, 0.001 µg/ml, 0.0001 µg/ml and 0 µg/ml. 25 µL/w cell suspension was added containing either 50.000 c/well HEK293/hCD27 cells or PBMCs. 25 µL/w rhCD70-FLAG was added (SEQ ID NO: 37) at 0.2 µg/ml final concentration. The cells incubated 1h on ice. Relevant isotype was included. 150 µL/w FACS buffer was added, the plate was centrifuged 300 g, 5 min. The supernatant was discarded. 200 µL/w FACS buffer was added and the plate was centrifuges at 300 g, 5 min. Finally the cells were resuspended in 50 µL/w secondary Ab (anti-FLAG mAb, cat. no. AD059F, Perkin Elmer, Massachusetts, USA) diluted 1:200 in FACS buffer. The cells were incubated on ice for 30 mins. The washing procedure was repeated. Cells were resuspended in 100 µL PBS. A

dead/live marker was included in control wells in order to ensure gating on living cells (7AAD, Cat. no. 559925, BD Biosciences, NJ, USA). The cells were analysed by flow cytometry on FACS Canto (BD Biosciences, NJ, USA). Cells alone, rhCD70-FLAG alone, secondary antibody alone and relevant isotype controls were included as controls.

- 5 Antibodies and Fabs were titrated from 10 µg/ml to 0.0001 µg/ml in a 10-fold dilution row and plotted by using GraphPad Prism version 6. Curves were fitted by using a nonlinear four parameter fit. The concentration in nM that resulted in 50% inhibition of rhCD70-FLAG binding to HEK293/hCD27 cells was calculated from the curves (Table 7). Fab 2F2 was identified as the most potent inhibitor. When comparing 2F2 hlgG4 with a Fab fragment
10 thereof, around 2-3 fold potency was lost. Four Fabs derived from the rabbit (F14, F15, F2 and F22) were identified as inhibitory.

Table 22: IC50 values of a-hCD27 Fabs and mAbs. competition with rhCD70

Clone ID	Enzymatically cleaved Fab	Rec. hlgG4 chimeric Fab	Rec hlgG4 chimeric mAb
2F2	0.4 nM	0.2 nM	0.12 nM
5F24	Non competing		
5F32	0.9 nM		
10F13	1.4 nM		
10F31	0.7 nM		
11F26	14 nM		
Fab 151		0.7 nM	
Fab 152		0.5 nM	
Fab 153		*	
Fab 154		*	
Fab 155		*	
Fab 156		*	

*Curves did not reach 100% inhibition and were not sigmoidal, hence fitting was not possible and no IC50 value could be calculated.

15

Hz and WT 5F24 and WT 2F2 mAb and Fabs were compared with prior art antibodies. The corresponding IC50 values are depicted in Table 23.

Table 23

NNCD no mAb	Clone ID	hlgG4 mAb
0307-0000-0081-1A	Celldex 1f5	2.6 nM
0307-0000-0082-1A	Celldex 1g5	*
0307-0000-0083-1A	Celldex 1h8	2.4 nM
0307-0000-0084-1A	Celldex 3h8	*
0307-0000-0085-1A	Celldex 3h12	2.2 nM
0307-0000-0086-1A	Celldex 2c2**	*
0307-0000-0087-1A	Celldex 3a10	*
0307-0000-0088-1A	Celldex 2g9**	2.3 nM
0307-0000-0076-2B	5F24	*
0307-0000-0050-2B	2F2	0.5 nM

*Curves did not reach 100% inhibition and were not sigmoidal, hence fitting was not possible and no IC50 value could be calculated.

5 Example 29

The humanized 5F24 Fab, 0307-0000-0198, was tested in CD27 reporter gene assay side-by-side with the commercially available LT27 (Cat. No. AB1091, Abcam). The 5F24 Fab has a more efficient inhibition, especially at lower concentrations, than the LT27 antibody. The inhibition reaches a plateau at about 80 % inhibition.

10

Table 24 The effect of humanized 5F24 Fab and LT27 antibody are defined as inhibition percentage of the activation of the CD27 reporter gene assay by 1 µg/ml CD70.

	0.02 µg/ml	0.2 µg/ml	2 µg/ml	20 µg/ml
0307-0000-0198 Hz5F24 Fab	24 %	60 %	72 %	78 %
LT27 Ab	11 %	49 %	66 %	77 %

In the humanized 5F24 Fab backmutations, V5Q in the Heavy Chain and K45Q in the Light Chain were introduced and in addition a free cysteine in either N152C or G157C positions. These variants, conjugated and unconjugated, were also tested in CD27 reporter gene assay. The conjugated variants almost retained full potency.

15

Table 24. The effect of humanized 5F24 Fab and LT27 antibody are defined as inhibition percentage of the activation of the CD27 reporter gene assay by CD70 expressing cells.

	0.02 µg/ml	0.2 µg/ml	2 µg/ml	20 µg/ml
0307-0000-0185 5F24 Fab	31%	79 %	83 %	87 %
H5F24-V5Q-K45Q-N152C 0307-0000-0351	23 %	65 %	71 %	78 %
H5F24-V5Q-K45Q-N152C-conjugated 0307-0000-0381	15 %	62 %	69 %	73 %
H5F24-V5Q-K45Q-G157C 0307-0000-0360	25 %	65 %	71 %	79 %
H5F24-V5Q-K45Q-G157C-conjugated 0307-0000-0382	12 %	57 %	64 %	67 %

5 While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

CLAIMS

1. A monovalent antibody which is capable of specifically binding CD27 and reducing CD27 induced signalling, said antibody being capable of inhibiting B-cell IgG secretion by at least 50%.

2. The monovalent antibody according to claim 1, said monovalent antibody having an IC50 value that is less than 10nM.

3. The monovalent antibody according to any one of claims 1-2, which does not compete with CD70 for binding to CD27.

4. The monovalent antibody according to any one claims 1-3, wherein said antibody reduces CD27/CD70 mediated co-stimulation of CD4+ and/or CD8+ T cells by more than 50%.

5. The monovalent antibody according to claim 1, which comprises a heavy chain having:
a CDRH1 sequence of amino acid residues 31 to 35 (DYAMH) of SEQ ID NO: 38, wherein one of these amino acid residues may be substituted by a different amino acid residue; and/or

a CDRH2 sequence of amino acid residues 50 to 66 (VISTYNGNTNYNQKLKG) of SEQ ID NO: 38, wherein one, two or three of these amino acids may be substituted by a different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 108 (DNGGTYGFTY) of SEQ ID NO: 38, wherein one, two or three of these amino acid residues may be substituted by a different amino acid.

6. The monovalent antibody according to any one of claims 1-2, which further comprises a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (KASQDINSYLN) of SEQ ID NO: 39, wherein one, two or three of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (RANRLVD) of SEQ ID NO: 39, wherein one or two of these amino acid residues may be substituted with a different amino acid; and/or

a CDRL3 sequence of amino acid residues 89 to 97 (LQYDEFPYT) of SEQ ID NO: 39, wherein one or two of these amino acid residues may be substituted with a different amino acid.

- 5 7. The monovalent antibody according to claim 1, which comprises a heavy chain having:
a CDRH1 sequence of amino acid residues 31 to 35 (DYAMH);
a CDRH2 sequence of amino acid residues 50 to 66 (VISTYNGNTNYNQKLKG); and
a CDRH3 sequence of amino acid residues 99 to 108 (DNGGTYGFTY) of SEQ ID NO: 38;
and which comprises a light chain having
10 a CDRL1 sequence of amino acid residues 24 to 34 (KASQDINSYLN);
a CDRL2 sequence of amino acid residues 50 to 56 (RANRLVD); and
a CDRL3 sequence of amino acid residues 89 to 97 (LQYDEFPYT) of SEQ ID NO: 39.

8. A monovalent antibody that is capable of specifically binding CD27, wherein the heavy
15 chain of said antibody comprises:

a CDRH1 sequence of amino acid residues 31 to 35 (NFWMN) of SEQ ID NO: 40,
wherein one of these amino acid residues may be substituted by a different amino acid
residue; and/or

- 20 a CDRH2 sequence of amino acid residues 50 to 66 (MIHPDSETRLNQKFKD) of
SEQ ID NO: 40, wherein one, two or three of these amino acids may be substituted by a
different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 99 to 108 (LDNDYDALDY) of SEQ ID
NO: 40, wherein one, two or three of these amino acid residues may be substituted by a
different amino acid.

25

9. The monovalent antibody according to claim 8, further comprising a light chain having:

a CDRL1 sequence of amino acid residues 24 to 34 (HASQNFNVWLS) of SEQ ID
NO: 41, wherein one, two or three of these amino acid residues may be substituted with a
different amino acid; and/or

- 30 a CDRL2 sequence of amino acid residues 50 to 56 (KASNLHT) of SEQ ID NO: 41,
wherein one or two of these amino acid residues may be substituted with a different amino
acid; and/or

a CDRL3 sequence of amino acid residues 89 to 97 (QQGQSYPT) of SEQ ID NO:
41, wherein one or two of these amino acid residues may be substituted with a different
35 amino acid.

10. The monovalent antibody according to claim 1, which comprises a heavy chain having a CDRH1 sequence of amino acid residues 31 to 35 (NFWMN),
a CDRH2 sequence of amino acid residues 50 to 66 (MIHPDSETRLNQKFKD); and
5 a CDRH3 sequence of amino acid residues 99 to 108 (LDNDYDALDY)
of SEQ ID NO: 40; and which comprises a light chain having:
a CDRL1 sequence of amino acid residues 24 to 34 (HASQNFNVWLS),
a CDRL2 sequence of amino acid residues 50 to 56 (KASNLHT) and
a CDRL3 sequence of amino acid residues 89 to 97 (QQGQSYDWT) of SEQ ID NO: 41.

11. The monovalent antibody according to claim 1, which comprises a heavy chain having:
a CDRH1 sequence of amino acid residues 30 to 34 (SNSIS) of SEQ ID NO: 50,
wherein one of these amino acid residues may be substituted by a different amino acid
residue; and/or

15 a CDRH2 sequence of amino acid residues 49 to 64 (IIYFSGATNYANWAKG) of
SEQ ID NO: 50, wherein one, two or three of these amino acids may be substituted by a
different amino acid residue; and/or

a CDRH3 sequence of amino acid residues 96 to 112 (DYDSYDAVSTNLYAFDP) of
SEQ ID NO: 50, wherein one, two or three of these amino acid residues may be substituted
20 by a different amino acid.

12. The monovalent antibody according to claim 11, which further comprises a light chain
having:

a CDRL1 sequence of amino acid residues 24 to 34 (QASEIIYANLA) of SEQ ID NO:
25 51, wherein one, two or three of these amino acid residues may be substituted with a
different amino acid; and/or

a CDRL2 sequence of amino acid residues 50 to 56 (RASTLAS) of SEQ ID NO: 51,
wherein one or two of these amino acid residues may be substituted with a different amino
acid; and/or

30 a CDRL3 sequence of amino acid residues 89 to 100 (QQGYSSSNVDNP) of SEQ
ID NO: 51, wherein one or two of these amino acid residues may be substituted with a
different amino acid.

13. The monovalent antibody according to claim 1, which comprises a heavy chain having:
35 a CDRH1 sequence of amino acid residues 30 to 34 (SNSIS); and

a CDRH2 sequence of amino acid residues 49 to 64 (IIYFSGATNYANWAKG) and
a CDRH3 sequence of amino acid residues 96 to 112 (DYDSYDAVSTNLYAFDP) of SEQ ID
NO: 50;

and which comprises a light chain having:

- 5 a CDRL1 sequence of amino acid residues 24 to 34 (QASEIIYANLA),
a CDRL2 sequence of amino acid residues 50 to 56 (RASTLAS) and
a CDRL3 sequence of amino acid residues 89 to 100 (QQGYSSSNVDNP) of SEQ ID NO:
51.

- 10 14. The monovalent antibody according to any one of claims 1 to 4 having a heavy chain and
light chain as defined in any one of SEQ ID NO's 60 to 72.

- 15 15. The monovalent antibody according to any one of claims 1 to 14, wherein the sequence
of amino acid residues of SEQ ID NO's 38 to 72 comprise or is modified to comprise one or
both backmutations V5Q in the Heavy Chain and K45Q in the Light Chain.

16. The monovalent antibody according to any one of claims 1 to 4 having the variable
domains hz5F24VH+V5Q, hz5F24VL+K45Q.

- 20 17. A fusion protein or conjugate comprising the monovalent antibody according to any one
of claims 1-16 and a half-life extending moiety.

18. The monovalent antibody according to any one of claims 1-16 or the fusion protein or
conjugate according to claim 17 for use as a medicament.