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DESCRIPTION

[0001] The present invention relates to humanized antibodies binding CD28, to monovalent fragments thereof, and to their therapeutic uses, in particular in the context of regulating T cell activation.

[0002] Abnormal activation of T cells is involved in the pathogenesis of many autoimmune diseases, and also in transplant rejection phenomena, where they cause an immune response directed against the transplanted organ to develop.

[0003] One of the most important systems for regulating T lymphocyte activation is the molecular system B7/CD28/CTLA4. This system plays, for example, an essential role in the mechanisms of transplant rejection (WOODWARD et al., Transplantation, 66, 14-20, 1998). The molecules B7.1 (CD80) and B7.2 (CD86) borne by the APCs can activate the receptor CD28 and also the receptor CTLA4 of T lymphocytes. The activation of CD28 sends the T lymphocyte a positive signal which stimulates the cell; on the other hand, the activation of CTLA4 sends a negative signal which leads to a non-response (anergy) (FALLARINO et al., J. Exp. Med., 188, 205-210, 1998).

[0004] Resting T lymphocytes express a large amount of CD28 and very little CTLA4. When there is a first cognitive contact between an APC and a T lymphocyte, the CD28/B7 interaction is favored, which activates the cell. It is only several hours after the initiation of activation that, due to the increase in membrane expression of CTLA4, the affinity of which for B7 is 5 to 10 times greater than that of CD28, the B7/CD28 interaction shifts in favor of a B7/CTLA4 interaction.

[0005] Regulatory T lymphocytes express a large amount of CD28 and of CTLA4 that prevent or allow, respectively, the suppressive activity of regulatory T lymphocytes. In the presence of APC expressing high level of B7, the CD28/B7 interaction prevents the suppressive activity of regulatory T lymphocytes (Sansom et al., Trends Immunol. 24, 314-319, 2003).

[0006] Selective inhibition of the agonist signal given to the T cell by CD28, leaving the antagonist system consisting of the pair CTLA4/B7 intact, via specific blocking of the CD28/B7 interaction, would make it possible to prevent T lymphocyte activation and to promote immune suppression by regulatory T lymphocytes. Such specific blocking of the CD28/B7 interaction can be obtained using some antibodies directed against CD28.

[0007] These antibodies are to be used in a monovalent form (for instance as Fab or scFv fragments), since when used in their divalent native form, their binding to CD28 brings about the dimerization and the activation of this receptor. Fab fragments each contain a light chain and the first half of a heavy chain; scFv fragments consist of the variable portions of the heavy and light chains of a parent antibody, connected to one another via a flexible linker (CLACKSON et al., Nature, 352, 624-628, 1991), thus forming a single-chain protein.

[0008] One such antibody is antibody CD28.3, produced by the hybridoma cell line CNCM I-2582, and disclosed in PCT application WO 02/051871. This antibody, when used in a monovalent form such as scFv fragments, is capable of blocking *in vitro* the CD28 receptor without activating it (PCT WO 02/051871; VANHOVE et al., Blood, 102, 564-70, 2003), and has shown also its efficiency *in vivo* in models of organ transplantation in mice and in primates (POIRIER et al., World Transplant Congress, Sydney, Australia. August 16-21, 2008; POIRIER et al, Sci Trans Med, 2:17, p17ra10, 2010).

[0009] A drawback of all monoclonal antibodies derived from murine sources, is their immunogenicity when administered to human subjects. They provoke anti-mouse immune response, which results in a lesser efficiency of the treatment, in particular when repeated administration is required.

[0010] This drawback can, in principle, be avoided by the use of humanized antibodies. The aim of humanization is to obtain a recombinant antibody which has similar antigen-binding properties as the mouse monoclonal antibody from which the complementarity-determining regions (CDRs) sequences were derived, and which is far less immunogenic in humans.

[0011] The CDRs are the portions of the variable domains of an antibody which directly contact the antigen and determine the antigen-binding specificity; the framework regions (FRs) which are located between the CDRs in the variable domains do not directly contact the antigen, but serves as a scaffold to maintain the global structure of the variable domains.

[0012] Several approaches to antibody humanization have been reported. The more widely used are based on "CDR grafting", which involves the transplantation of the CDRs of a murine antibody into appropriate human FRs. However, in many antibodies, some FR residues are important for antigen binding, because they influence the conformation of CDRs and thus their antigen binding properties, in particular the binding affinity. A loss in binding affinity is particularly detrimental in the case of an antibody intended to be used in a monovalent form which generally exhibit less affinity for the antigen than the native divalent antibody. Thus, in most cases, it is further necessary, in order to obtain a sufficient binding affinity, to reintroduce one or several framework residues from the mouse antibody in the human FRs, with the risk of simultaneously bringing back unwanted immunogenicity.

[0013] Another approach to antibody humanisation, called "de-immunization", involves the identification within the FRs regions of the antibody, of B-cell and T-cell epitopes recognized as "foreign" and therefore potentially immunogenic in humans, and to remove them by appropriate amino-acids substitutions. This approach however also entails the risk that FR residues important for antigen binding are deleted. Moreover, some immunogenic epitopes may lie in the CDRs and trying to remove them involves a very high risk of destroying not only the antigen-binding affinity but also the antigen-binding specificity of the antibody.

[0014] Therefore, a major issue in antibody humanisation is to determine which amino acid

residues are critical for retaining the antigen-binding properties. Various methods have been proposed for predicting the more appropriate sites for substitution in the FRs regions. Although they provide general principles that may be of some help in the first steps of humanization, the final result greatly varies from an antibody to another. Thus, for a given antibody, it is very difficult to foretell which substitutions will provide the desired result. In the case wherein not only substitutions in the FRs, but also in the CDRs would be necessary to decrease satisfactorily the immunogenicity in humans, the final result becomes totally unpredictable.

[0015] Tan et al. Blood 96:11, 31A, 2000 discloses a method for producing a functional humanized anti-CD28 antibody,

[0016] Saldanha: "Chapter 6: Molecular Engineering I: Humanization" In: Stephan Duebel: "Handbook of Therapeutic Antibodies", Wiley-VCH, 119-144, 2007 discloses methods of humanizing antibodies.

[0017] WO 2010/009391 discloses domain antibodies which consist of or comprise a single immunoglobulin variable domain that specifically binds and antagonizes the activity of CD28.

[0018] WO 2010/082136 discloses recombinant monovalent antibodies which are heterodimers of a first protein chain comprising the variable domain of the heavy chain of an antibody of interest and the CH2 and CH3 domains of an IgG immunoglobulin and a second protein chain comprising the variable domain of the light chain of said immunoglobulin of interest and the CH2 and CH3 domains of said IgG immunoglobulin.

[0019] WO 2010/070047 discloses soluble CD47 binding polypeptides, for use as a medicament, in particular for the prevention or treatment of autoimmune and inflammatory disorders.

[0020] The inventors have succeeded in producing humanized CD28.3 (hereinafter referred to as hCD28.3), with a low immunogenicity, and which, although it has several amino acids substitutions including a non-conservative K→Q substitution in the CDR2 of the heavy chain, retains the CD28 binding properties of the parent mouse CD28.3. When used in a monovalent form, the hCD28.3 of the invention also retains the CD28 binding properties of the parent mouse CD28.3.

The present invention provides an anti-CD28 antibody having a CD28-binding site consisting of:

- a first variable domain (also defined herein as the "heavy chain variable domain") defined by the following sequence:
VQLQQSGAELKKPGASVKVSCASGYTFTEYIIHWIKLRSGQGLEWI
GWFYPGSNDIQYNAQFKGKATLTADKSSSTVYMELTGLTPEDSAVYFCARRDDFSG
YDALPYWGQGTLVTVSA (SEQ ID NO: 1), wherein said variable domain may optionally further comprise a Q residue at its N-terminal end;
- a second variable domain (also defined herein as the "light chain variable domain")

defined by the following sequence:

DIQMTQSPSSLSASVGDRVITCKTNENIYSNLAWYQQKDGKSPQLL

IYAATHLVEGVPSRFSGSGSGTQYSLTISSLQPEDFGNYQCQHFWGTPXTFGGGTKLEI

KR, wherein X= C or A.

[0021] Also disclosed herein is an antibody having a CD28-binding site consisting of:

- a first variable domain having the CDRs of the variable domain of SEQ ID NO: 1;
- a second variable domain having the CDRs of the variable domain of SEQ ID NO: 2.

[0022] The term "anti-CD28 antibody" herein refers to any antigen-binding protein having at least one antigen-binding site (consisting of the variable domains of the light chain and of the heavy chain) able to specifically bind human CD28. It encompasses antibodies in a divalent form (such as native immunoglobulin molecules or F(ab)'2 fragments) with two CD28-binding sites, as well as antibodies in a monovalent form which have a single CD28-binding site, (for instance Fab, Fab', Fv and scFv fragments). In most cases, antibodies in a monovalent form will be preferred.

[0023] It includes in particular recombinant antibodies comprising a CD28-binding site associated with one or more heterologous polypeptide(s).

[0024] By way of example, an antibody of the present disclosure may be a recombinant Fab or Fab' fragment containing the constant domain CH1 of a human immunoglobulin fused at the C-terminal end of the variable domain of SEQ ID NO: 1, and the constant domain CL of a human immunoglobulin fused at the C-terminal end of the variable domain of SEQ ID NO: 2. An example of such a recombinant Fab fragment is a Fab fragment with a heavy chain having the sequence of amino-acids 21-251 of SEQ ID NO: 4 and a light chain having the sequence of amino-acids 21-234 of SEQ ID NO: 6.

[0025] Also, a hCD28.3 antibody may comprise, besides the variable domains of SEQ ID NO: 1 and SEQ ID NO: 2, defined above, one or more of the following components:

- a human constant region (Fc). This constant region can be selected among constant domains from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Preferred constant regions are selected among constant domains of IgG, in particular IgG4.
- a protein which makes it possible to prolong the plasma half-life when it is administered *in vivo* under monovalent form as disclosed for instance in PCT WO 02/051871; in a preferred embodiment, said protein is the CH2-CH3 domains of an IgG molecule, as disclosed in PCT/IB/2010/000196; according to said embodiment, a hCD28.3 monovalent antibody is an heterodimer of:

- a first protein chain consisting essentially of, from its N-terminus to its C-terminus:
 - * a region A having the sequence SEQ ID NO: 1;
 - * a region B consisting of a peptide linker and the CH2 and CH3 domains of an IgG immunoglobulin;
- a second protein chain consisting essentially of, from its N-terminus to its C-terminus:
 - * a region A' having the sequence SEQ ID NO: 2;
 - * a region B identical to the region B of the first polypeptide.

[0026] Preferably, the peptide linker is the hinge region of human IgG 1 immunoglobulins having the sequence EPKSCDKTHTCPPCP (SEQ ID NO: 7), and the CH2 and CH3 domains are those of an immunoglobulin of the IgG4 subclass. One can also use a shortened version of said hinge region, having the sequence DKTHTCPPCP (SEQ ID NO: 8).

[0027] According to a preferred embodiment, the polypeptide sequence of the first protein chain is the sequence of amino-acids 21-368 of SEQ ID NO: 10, and the polypeptide sequence of the second protein chain is the sequence of amino-acids 21-355 of SEQ ID NO: 12. According to another preferred embodiment, the polypeptide sequence of the first protein chain is the sequence of amino-acids 21-373 of SEQ ID NO: 14, and the polypeptide sequence of the second protein chain is the sequence of amino-acids 21-360 of SEQ ID NO: 16.

[0028] Optionally, a hCD28.3 antibody of the invention may further comprise one or more of the following components:

- a protein having pharmacological activity (for example a toxin);
- one or more tag polypeptide(s).

[0029] Alternatively, to prolong their plasma half life, in particular when they are under the form of Fab fragments, the antibodies of the invention can be conjugated with water soluble polymers such as polyethylene glycol (PEGylation). PEGylation is a classical way to enhance the pharmacokinetic properties of therapeutic polypeptides, and can be achieved by techniques known in the art.

[0030] In this respect, the inventors found that the replacement of the original cysteine residue at position 96 of the variable domain of the native CD 28.3 by an alanine or an asparagine residue (resulting in an antibody having a light chain containing a variable domain of SEQ ID NO: 2 wherein X = A or N) allowed a better efficacy in pegylation of the antibody using maleimide-activated polyethylene glycol (targeting reactive cystein residues), without modifying substantially its binding activity, although cysteine-96 is comprised in the CDR3 of the antibody

light chain. The benefit of the replacement of the original cysteine residue at position 96 of the variable domain mainly consist in a specific branching of the polyethylene glycol onto the C-terminal cysteine residue of the heavy chain. Without replacement of the original cysteine residue at position 96 of the variable domain of the native CD 28.3, maleimide-activated polyethylene glycol can bind to that cysteine residue and impair the binding activity of the Fab molecule.

The inventors also found that addition of a di-alanine extension after the C- terminal cysteine of the heavy chain also resulted in a better pegylation efficiency.

[0031] The invention provides a polynucleotide encoding the antibody of the invention.

[0032] Also disclosed is a polynucleotide selected among:

1. a) a polynucleotide encoding a polypeptide having the CDRs of SEQ ID NO: 1, in particular a polynucleotide encoding a polypeptide of SEQ ID NO: 1;
2. b) a polynucleotide encoding a polypeptide having the CDRs of SEQ ID NO: 2, in particular a polynucleotide encoding a polypeptide of SEQ ID NO: 2;
3. c) a polynucleotide encoding an hCD28.3 antibody of the invention, as defined above.

[0033] Polynucleotides of the invention generally also comprise additional sequences: for instance they may advantageously comprise a sequence encoding a leader sequence or signal peptide allowing secretion of said protein chain.

[0034] The present disclosure also encompasses recombinant vectors, in particular expression vectors, comprising a polynucleotide of the invention, associated with transcription- and translation-controlling elements which are active in the host cell chosen. Vectors which can be used to construct expression vectors in accordance with the disclosure are known in themselves, and will be chosen in particular as a function of the host cell intended to be used.

[0035] The present disclosure also encompasses host-cells transformed with a polynucleotide of the invention. Preferably, said host cell is transformed with a polynucleotide comprising a sequence encoding the heavy chain of a hCD28.3 antibody of the invention and a polynucleotide comprising a sequence encoding the light chain of a hCD28.3 antibody of the invention, and expresses said antibody. Said polynucleotides can be inserted in the same expression vector, or in two separate expression vectors.

[0036] Host cells which can be used in the context of the present invention can be prokaryotic or eukaryotic cells. Among the eukaryotic cells which can be used, mention will in particular be made of plant cells, cells from yeast, such as *Saccharomyces*, insect cells, such as *Drosophila* or *Spodoptera* cells, and mammalian cells such as HeLa, CHO, 3T3, C127, BHK, COS, etc., cells.

[0037] The construction of expression vectors of the disclosure and the transformation of the

host cells can be carried out by the conventional techniques of molecular biology.

[0038] Still another object of the disclosure is a method for preparing a hCD28.3 antibody of the invention. Said method comprises culturing a host-cell transformed with a polynucleotide comprising a sequence encoding the heavy chain of a hCD28.3 antibody of the invention and a polynucleotide comprising a sequence encoding the light chain of a hCD28.3 antibody of the invention and recovering said antibody from said culture.

[0039] If the antibody is secreted by the host-cell, it can be recovered directly from the culture medium; if not, cell lysis will be carried out beforehand. The antibody can then be purified from the culture medium or from the cell lysate, by conventional procedures, known in themselves to those skilled in the art, for example by fractionated precipitation, in particular precipitation with ammonium sulfate, electrophoresis, gel filtration, affinity chromatography, etc.

[0040] The hCD28.3 antibodies of the invention can be used to obtain medicinal products. These medicinal products are also part of the object of the invention.

[0041] The present invention also comprises a therapeutic composition comprising a hCD28.3 antibody of the invention, together with a pharmaceutically acceptable excipient.

[0042] Preferably, said composition is a composition for parenteral administration, formulated to allow the administration of a dose of from 0.5 to 20 mg/Kg, advantageously of from 5 to 10 mg/Kg of an hCD28.3 antibody of the invention. The injection route of the composition can be preferably sub-cutaneous or intra-venous.

[0043] For instance, hCD28.3 antibodies of the invention can be used to obtain immunosuppressant medicinal products which selectively blocks T cell activation phenomena involving the CD28 receptor. Such immunosuppressant medicinal products which act by selective blocking of CD28 have applications in all T lymphocyte-dependent pathological conditions, including in particular transplant rejection, graft-versus-host disease, T lymphocyte-mediated autoimmune diseases, such as type I diabetes, rheumatoid arthritis or multiple sclerosis, and type IV hypersensitivity, which is involved in allergic phenomena and also in the pathogenesis of chronic inflammatory diseases, in particular following infection with a pathogenic agent (in particular leprosy, tuberculosis, leishmaniasis, listeriosis, etc.).

[0044] The present invention will be understood more clearly from the further description which follows, which refers to nonlimiting examples of the preparation and properties of a hCD28.3 antibody in accordance with the invention.

[0045] The construction of expression vectors of the disclosure and the transformation of host-cells can be made by the standard techniques of molecular biology.

[0046] A hCD28.3 antibody of the invention can be obtained by culturing a host cell containing an expression vector comprising a nucleic acid sequence encoding said antibody, under

conditions suitable for the expression thereof, and recovering said antibody from the host cell culture.

[0047] The present invention will be further illustrated by the following additional description, which refers to examples illustrating the properties of hCD28.3 antibodies of the invention. It should be understood however that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

LEGENDS OF THE DRAWINGS

[0048]

Figure 1: Nucleotidic and amino acid sequences of the Signal-VH-hCHI construction. Bold: leader sequence; Underlined: positions of the CDRs of the parent CD28.3 antibody. Italics: human CHI region; Highlighted and double underlined: substitutions made in the CD28.3 antibody VH region.

Figure 2: Nucleotidic and amino acid sequences of the Signal-VL-hCK construction. Bold: leader sequence; Underlined: positions of the CDRs of the parent CD28.3 antibody. Italics: human c kappa region; Highlighted and double underlined: substitutions made in the CD28.3 antibody VL region.

Figure 3: A) optical density at 405 nm for increasing concentrations of FR104, hCD28.3 Fab or CD28.3 Fab in the Binding ELISA; B) calculation of the regression curves, allowing for determining comparative AC50 values.

Figure 4: Nucleotidic and amino acid sequences of the hVHCD28.3-short hingeγ1-hγ4CH2CH3 construction. Bold: leader sequence. Underlined: CDRs. Double underlined: hinge region. Dotted underlined: CH2-CH3 domains of the human IgG4.

Figure 5: Nucleotidic and amino acid sequences of the hVLCD28.3-short hingeγ1-hγ4CH2CH3 construction. Bold: leader sequence. Underlined: CDRs. Double underlined: hinge region. Dotted underlined: CH2-CH3 domains of the human IgG4.

Figure 6: Nucleotidic and amino acid sequences of the hVHCD28.3-full hingeγ1-hγ4CH2CH3 construction. Bold: leader sequence. Underlined: CDRs. Double underlined: hinge region. Dotted underlined: CH2-CH3 domains of the human IgG1.

Figure 7: Nucleotidic and amino acid sequences of the hVLCD28.3-full hingeγ1-hγ4CH2CH3 construction. Bold: leader sequence. Underlined: CDRs. Double underlined: hinge region. Dotted underlined: CH2-CH3 domains of the human IgG1.

Figure 8: Anti-CD28 binding properties of hVH/VL CD28.3 monovalent antibodies. COS cells were co-transfected with 2 µg (each) pSignal-hVH-short hingeγ1-hγ4CH2-CH3 and pSignal-hVL-short hingeγ1-hγ4CH2-CH3, or co-transfected with 2 µg (each) pSignal-hVH-full hingeγ1-

hy4CH2-CH3 and pSignal-hVL-full hinge_{y1}-hy4CH2-CH3. After 6 days, supernatants were collected and monovalent antibodies were dosed using a first sandwich ELISA. Supernatants were also assessed with a binding ELISA on immobilized CD28 target molecules and bound monovalent anti-CD28 antibodies were revealed with anti-human Fc antibodies labeled with peroxidase. A: Optical density obtained with indicated molecules according to their concentration. B: table with regression curves and the calculation of ED₅₀ (effective dose 50), the concentration needed to reach 50% binding activity in this assay.

Figure 9: hVH/VL CD28.3 monovalent antibodies inhibit IL-2 secretion by activated T cells. Jurkat T cells were stimulated with SEE superantigen and Raji antigen-presenting-cells during 48h, in the presence of indicated concentrations of purified hVH/VL-short hinge_{y1}-hy4CH2-CH3 monovalent antibodies. Supernatant were collected and IL-2 measured by ELISA.

Figure 10: SP sepharose HP-chromatography (left) and SDS-PAGE (right) under unreduced conditions after pegylation of C96-Fabs from humanised CD28.3 antibody. Lane 1: marker; lane 2:load; lane 3: Peak 1; lane 4: Peak 2; lane 5: Peak 3.

Figure 11: Binding properties for CD28 of recombinant hCD28.3 Fabs with or without C96 mutations. The graph shows binding activity (Y axis) according to Fab concentration (X axis).

Figure 12: SP sepharose HP-chromatography (left) and SDS-PAGE (right) under unreduced conditions after pegylation of C96A-Fabs from humanised CD28.3 antibody. Lane 1: MW markers; lane 2: Pegylated proteins pre-chromatography; lane 3: peak 1 containing the monopegylated Fab, representing 41% of the starting material.

Figure 13: SP sepharose HP-chromatography (left) and SDS-PAGE (right) under unreduced conditions after pegylation of C96A-Fabs from humanised CD28.3 antibody with a CAA C-terminal sequence in the heavy chain. Lane 1: MW markers; lane 2: Pegylated proteins pre-chromatography; lane 3: peak.

EXAMPLE 1: CONSTRUCTION AND EUCARYOTIC EXPRESSION OF A hCD28.3 MONOVALENT ANTIBODY (Fab FRAGMENT)

Heavy chain:

[0049] The sequence encoding the VH region of hCD28.3 (SEQ ID NO: 1) in fusion with the sequence encoding the human CH1 region (NCBI Accession number AAF03881) and with a sequence encoding the leader peptide of the heavy chain of the native murine CD28.3 antibody, was synthesized chemically, and introduced in the cloning vector pGA18 (Geneart) for amplification. The sequence was then excised by digestion with KpnI/BamHI restriction enzymes and subcloned into the KpnI/BamHI sites of the plasmid pcDNA3.1-hygro (Invitrogen).

Positive clones were amplified and purified by Midiprep-endotoxin free (Macherey-Nagel) for transfection step.

[0050] The resulting plasmid is designated pSignal-VH-hCH1. It comprises a construct containing the sequence encoding the VH region of hCD28.3 between the sequence encoding the CD28.3 heavy chain leader peptide and the sequence encoding the human CH1 region (NCBI Accession number AAF03881). The nucleotidic and amino acid sequences of this construct are shown on Figure 1. They are also represented as SEQ ID NO: 3 and SEQ ID NO: 4 in the enclosed sequence listing.

Light chain:

[0051] The sequence encoding the VL region of hCD28.3 (SEQ ID NO: 2) in fusion with the sequence encoding the human c kappa region (NCBI accession number BAC01725) and with a sequence encoding the leader peptide of the light chain of the native murine CD28.3 antibody, was synthesized chemically, and introduced in the cloning vector pGA18 (Geneart) for amplification. The sequence was then excised by digestion with KpnI/BamHI restriction enzymes and subcloned into the KpnI/BamHI sites of the plasmid pcDNA3.A-hygro (Invitrogen). Positive clones were amplified and purified by Midiprep-endotoxin free (Macherey-Nagel) for transfection step.

[0052] The resulting plasmid is designated pSignal-VL-hCk. It comprises a construct containing the sequence encoding the VL region of hCD28.3 between the sequence encoding the CD28.3 light chain signal peptide and the sequence encoding the human c kappa region (NCBI accession number BAC01725). The nucleotidic and amino acid sequences of this construct are shown on Figure 2. They are also represented as SEQ ID NO: 5 and SEQ ID NO: 6 in the enclosed sequence listing.

Eucaryotic expression

[0053] COS cells were co-transfected with 2 µg (each) pSignal-VL-hCHI and pSignal-VH-hCH1 using the Eugene lipofection kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Cultures were maintained for 3 days at 37°C, divided one third, and put back into culture for an additional 3 days, after which time the cell supernatants were collected.

[0054] The activity of the hCD28.3 monovalent antibody is evaluated directly in the supernatant by ELISA, as described in Example 2 below.

EXAMPLE 2: DETECTION OF THE hCD28.3 Fab FRAGMENT BINDING ACTIVITY BY ELISA

[0055] The binding properties of the hCD28.3 Fab fragment have been compared with those obtained after transfection of Cos cells with plasmids coding for CD28.3 Fab (not humanized), using two ELISA assays

* First (Sandwich ELISA), the concentrations of the hCD28.3 and CD28.3 Fab fragments in the culture supernatants of transfected COS cells have been determined using a sandwich ELISA. Briefly, the anti-CD28 Fab contained in the supernatants are first captured by a rabbit polyclonal antibody, specific for the heavy and light variable domains of CD28.3 (obtained after immunization of rabbits with a single-chain-Fv containing the heavy and light variable domains of the native CD28.3, and purified by immunoabsorption on CD28.3 Fab-Sepharose) . The captured proteins are then revealed with a murine monoclonal antibody directed to the kappa chain of human IgG, followed by a polyclonal goat anti-mouse antibody labelled with peroxidase. Bound antibody was revealed by colorimetry using the TMB substrate, and read at 405 nm.

The OD corresponding to different dilutions of the supernatant are then compared to a standard curve obtained with known quantities of a CD28.3 Fab, called FR104, purified from culture supernatant of transformed CHO cells with standard techniques of chromatography, and dosed with a BCA (bisynchronous acid) assay. FR104 contains the native (not humanized), VH and VL regions of the CD28.3 antibody. Therefore, we can evaluate the amount of Fab proteins present in cell supernatants.

* Second (Binding ELISA), for testing the binding activity of hCD28.3 Fab fragments compared to CD28.3 Fab, chimeric human CD28/Fc (R&D Systems, Abingdon, United Kingdom) was used at 2 µg/ml in carbonate buffer 0.05M pH 9.2 to coat the wells (50 µL/well) of microtiter plates (Nunc Immunoplates) overnight at 4°C. These immobilized CD28 target molecules will bind only immunoreactive molecules with anti-CD28 activity.

[0056] The wells were then washed 3 times successively with 200 µL PBS-0.05% Tween, and saturated with 100µL PBS Tween 0.1% BSA 1% for 2 hours at 37°C.

[0057] Then, after 3 washings with 200 µL PBS-0.05%Tween, supernatants containing known concentrations of CD28.3 or hCD28.3 Fab fragments were added (50 µL/well) at different dilutions in PBS-0.1% Tween and incubated for 2 hours at 37°C. After 3 washings with 200 µL PBS-0.05%Tween, a murine monoclonal antibody directed to the kappa chain of human IgG, (1/10000 dilution) was added (1 hour, 37°C), followed by peroxidase-conjugated goat anti-mouse antibodies (1/2000 dilution), followed by colorimetric revelation using the TMB substrate and reading at 405 nm.

[0058] Then the results are plotted as the absorbance (Y axis), measured with the binding ELISA, according to the Fab concentration (X axis), measured with the sandwich ELISA. An AC50 (Antibody Concentration 50) is determined after calculating the slope of the curve in its linear range as the concentration of the anti-CD28 Fab needed to reach 50% of the maximal

optical density (OD) in the binding assay.

[0059] The results are shown on Figure 3 and Table I.

[0060] Figure 3A shows the optical density at 405 nm for increasing concentrations of FR104, hCD28.3 Fab or CD28.3 Fab in the Binding ELISA.

[0061] Figure 3B shows the calculation of the regression curves, allowing for determining comparative AC50 values.

[0062] Table I below summarises the OD50, the equation, and the AC50 for the standard FR104, and the Fab fragments VH-wild type + VL-wild type and Fab hCD28.3

Table I

	OD50	Equation	AC50
Std FR104	1,792	$y = 1,1424 \ln(x) - 3,6351$	115
CD28.3 Fab	1,82	$y = 0,9776 \ln(x) - 3,2483$	162
hCD28.3 Fab	1,804	$y = 1,0217 \ln(x) - 3,2859$	151

[0063] These results show that 50% of the binding activity to CD28 could be reached at a concentration similar for Fab fragments VH- wild type + VL- wild type (CD28.3 Fab) and hCD28.3 Fab. The concentration is slightly lower for the standard, probably because it is purified before the assay. Thus hCD28.3 retains the CD28-binding properties of the wild type VH and VL sequences of CD28.

EXAMPLE 3: CONSTRUCTION AND EUKARYOTIC EXPRESSION OF A hCD28.3 MONOVALENT ANTIBODY (FV-FC FRAGMENT) WITH A SHORT $\gamma 1$ HINGE AND A $\gamma 4$ CH2-CH3 DOMAIN

Heavy chain:

[0064] The sequence encoding the VH region of hCD28.3 (SEQ ID NO: 1) in C-terminal fusion with the sequence encoding a portion of the hinge region of the human IgG1 (SEQ ID NO: 8), with CH2-CH3 domains of the human IgG4 (nucleotides 787 to 1440 of the sequence NCBI Accession number BC025985) and in N-terminal position with a sequence encoding the leader peptide of the heavy chain of the native murine CD28.3 antibody, was synthesized chemically, and introduced in the cloning vector pMA (Geneart) for amplification. The sequence was then excised by digestion with NheI/EcoRI restriction enzymes and subcloned into the NheI/EcoRI sites of the plasmid pCneo (Promega). After transformation of *E. coli* cells, positive clones were amplified and extracted plasmids were purified by Midiprep-endotoxin free columns

(Macherey-Nagel).

[0065] The resulting plasmid is designated pSignal-hVH-shorthingey1-hy4CH2-CH3. It comprises a construct containing the sequence encoding the VH region of hCD28.3 between the sequence encoding the CD28.3 heavy chain signal peptide and the sequence encoding a part of the human γ 1 hinge region and of the human γ 4 CH2-CH3 domains. The nucleotidic and amino acid sequences of this construct are shown on Figure 4. They are also represented as SEQ ID NO: 9 and SEQ ID NO: 10 in the enclosed sequence listing.

Light chain:

[0066] The sequence encoding the VL region of hCD28.3 (SEQ ID NO: 2) in fusion with the sequence encoding a portion of the hinge region of the human IgG1 (SEQ ID NO: 8), with CH2-CH3 domains of the human IgG4 (nucleotides 787 to 1440 of the sequence NCBI Accession number BC025985) and in N-terminal position with a sequence encoding the leader peptide of the heavy chain of the native murine CD28.3 antibody, was synthesized chemically, and introduced in the cloning vector pMA (Geneart) for amplification. The sequence was then excised by digestion with NheI/EcoRI restriction enzymes and subcloned into the NheI/EcoRI sites of the plasmid pCINeo (Promega). After transformation of E. coli cells, positive clones were amplified and extracted plasmids were purified by Midiprep-endotoxin free columns (Macherey-Nagel).

[0067] The resulting plasmid is designated pSignal-hVL-shorthingey1-hy4CH2-CH3. It comprises a construct containing the sequence encoding the VL region of hCD28.3 between the sequence encoding the CD28.3 light chain signal peptide and the sequence encoding a part of the human γ 1 hinge region and of the human γ 4 CH2-CH3 domains. The nucleotidic and amino acid sequences of this construct are shown on Figure 5. They are also represented as SEQ ID NO: 11 and SEQ ID NO: 12 in the enclosed sequence listing.

Eukaryotic expression

[0068] COS cells were co-transfected with 1 μ g (each) pSignal-hVL-shorthingey1-hy4CH2-CH3 and pSignal-hVH-shorthingey1-hy4CH2-CH3, using the Lipofectamine lipofection kit (Invitrogen) according to the manufacturer's instructions. Cultures were maintained for 3 days at 37°C, after which time the cell supernatants were collected. The activity of the monovalent antibody is evaluated directly in the supernatant by ELISA, as described in Example 5 below.

EXAMPLE 4: CONSTRUCTION AND EUCARYOTIC EXPRESSION OF A hCD28.3 MONOVALENT ANTIBODY (FV-FC FRAGMENT) WITH A FULL LENGTH γ 1 HINGE AND A γ 4 CH2-CH3 DOMAIN

Heavy chain:

[0069] The sequence encoding the VH region of hCD28.3 (SEQ ID NO: 1) in C-terminal fusion with the sequence encoding a full length hinge region of the human IgG1 (SEQ ID NO: 7), with CH2-CH3 domains of the human IgG4 (nucleotides 787 to 1440 of the sequence NCBI Accession number BC025985) and in N-terminal position with a sequence encoding the leader peptide of the heavy chain of the native murine CD28.3 antibody, was synthesized chemically, and introduced in the cloning vector pMA (Geneart) for amplification. The sequence was then excised by digestion with NheI/EcoRI restriction enzymes and subcloned into the NheI/EcoRI sites of the plasmid pCIneo (Promega). After transformation of *E. coli* cells, positive clones were amplified and extracted plasmids were purified by Midiprep-endotoxin free columns (Macherey-Nagel).

[0070] The resulting plasmid is designated pSignal-hVH-fullhingey1-hy4CH2-CH3. It comprises a construct containing the sequence encoding the VH region of hCD28.3 between the sequence encoding the CD28.3 heavy chain signal peptide and the sequence encoding the human γ 1 hinge region and the human γ 4 CH2-CH3 domains. The nucleotidic and amino acid sequences of this construct are shown on Figure 6. They are also represented as SEQ ID NO: 13 and SEQ ID NO: 14 in the enclosed sequence listing.

Light chain:

[0071] The sequence encoding the VL region of hCD28.3 (SEQ ID NO: 2) in fusion with the sequence encoding the full length hinge region of the human IgG1 (SEQ ID NO: 7), with CH2-CH3 domains of the human IgG4 (nucleotides 787 to 1440 of the sequence NCBI Accession number BC025985) and in N-terminal position with a sequence encoding the leader peptide of the heavy chain of the native murine CD28.3 antibody, was synthesized chemically, and introduced in the cloning vector pMA (Geneart) for amplification. The sequence was then excised by digestion with NheI/EcoRI restriction enzymes and subcloned into the NheI/EcoRI sites of the plasmid pCIneo (Promega). After transformation of *E. coli* cells, positive clones were amplified and extracted plasmids were purified by Midiprep-endotoxin free columns (Macherey-Nagel).

[0072] The resulting plasmid is designated pSignal-hVL-fullhingey1-hy4CH2-CH3. It comprises a construct containing the sequence encoding the VL region of hCD28.3 between the sequence encoding the CD28.3 light chain signal peptide and the sequence encoding the human γ 1 full length hinge region and of the human γ 4 CH2-CH3 domains. The nucleotidic and amino acid sequences of this construct are shown on Figure 7. They are also represented as SEQ ID NO: 15 and SEQ ID NO: 16 in the enclosed sequence listing.

Eukaryotic expression

[0073] COS cells were co-transfected with 1 µg (each) pSignal-hVH-fullhingey1-hy4CH2-CH3 and pSignal-hVL-fullhingey1-hy4CH2-CH3 plasmids using the Lipofectamine lipofection kit (Invitrogen) according to the manufacturer's instructions. Cultures were maintained for 3 days at 37°C, after which time the cell supernatants were collected.

[0074] The activity of the hCD28.3 monovalent antibody is evaluated directly in the supernatant by ELISA, as described in Example 5 below.

EXAMPLE 5: EVALUATION OF THE HCD28.3-FULL LENGTH γ 1 HINGE- γ 4CH2-CH3 DOMAINS AND HCD28.3-SHORT γ 1 HINGE- γ 4CH2-CH3 DOMAINS MONOVALENT ANTIBODIES BINDING ACTIVITY BY ELISA

[0075] The binding properties of the hCD28.3 monovalent antibodies hCD28.3-full γ 1 hinge- γ 4CH2-CH3 domains and hCD28.3-short γ 1 hinge- γ 4CH2-CH3 domains produced by transfected COS cells have been analysed using two ELISA assays.

* First (Sandwich ELISA), the concentrations of the hCD28.3 monovalent antibodies in the culture supernatants of transfected COS cells have been determined using a sandwich ELISA. Briefly, the monovalent antibodies contained in the supernatants are first captured by a goat polyclonal antibody directed to human IgG. The captured proteins are then revealed with a biotinylated goat polyclonal anti-human IgG, Fc specific, antibody, then, a Peroxidase-conjugated streptavidin. Bound antibody was revealed by colorimetry using the TMB substrate, and read at 405 nm.

The OD corresponding to different dilutions of the supernatant are then compared to a standard curve obtained with known quantities of hCD28.3 monovalent antibodies, purified from culture supernatant of transformed CHO cells with standard techniques of chromatography, and dosed with a BCA (bisynchronic acid) assay.

* Second (Binding ELISA), for testing the binding activity of hCD28.3 monovalent antibodies, chimeric human CD28/Fc (R&D Systems, Abingdon, United Kingdom) was used at 2 µg/ml in carbonate buffer 0.05M pH 9.2 to coat the wells (50 µL/well) of microtiter plates (Nunc Immunoplates) overnight at 4°C. These immobilized CD28 target molecules will bind only immunoreactive molecules with anti-CD28 activity.

[0076] The wells were then washed 3 times successively with 200 µL PBS-0.05% Tween, and saturated with 100µL PBS Tween 0.1% BSA 1% for 2 hours at 37°C.

[0077] Then, after 3 washings with 200 µL PBS-0.05%Tween, supernatants containing known concentrations of the monovalent antibodies to be tested were added (50 µL/well) at different

dilutions in PBS-0.1% Tween and incubated for 2 hours at 37°C. After 3 washings with 200 µL PBS-0.05% Tween, we added (1/500 dilution; 1 hour, 37°C) a rabbit polyclonal antiserum, specific for the heavy and light variable domains of CD28.3 (obtained after immunization of rabbits with a single-chain-Fv containing the heavy and light variable domains of the native CD28.3, and purified by immunoadsorption on CD28.3 Fab-Sepharose). This was followed by peroxidase-conjugated donkey anti-rabbit antibodies (1/2000 dilution), followed by colorimetric revelation using the TMB substrate and reading at 405 nm.

[0078] Then the results are plotted as the absorbance (Y axis), measured with the binding ELISA, according to the monovalent antibody concentration (X axis), measured with the sandwich ELISA. An AC50 (Antibody Concentration 50) is determined after calculating the slope of the curve in its linear range as the concentration of the monoclonal antibody needed to reach 50% of the maximal optical density (OD) in the binding assay.

[0079] Figure 8 compares binding activities of hCD28.3-full IgG1 hinge-IgG4CH2-CH3 domains with hCD28.3-short IgG1 hinge-IgG4CH2-CH3 domains monovalent antibodies in the Binding ELISA (Figure 8A).

[0080] Figure 8B summarises the equation, the regression factor and the AC50 for the monovalent antibodies.

[0081] These results show that 50% of the binding activity to CD28 could be reached at a concentration similar for hCD28.3-full γ 1 hinge- γ 4CH2-CH3 domains or hCD28.3-short γ 1 hinge- γ 4CH2-CH3 domains monovalent antibodies.

EXAMPLE 6: hCD28.3 MONOVALENT ANTIBODIES PREVENTS T CELL ACTIVATION

[0082] To verify that hCD28.3 monovalent antibody blocks CD28-dependent T cell activation, we stimulated human T cells (Jurkat cells) with SEE superantigen presented by a Raji B cell line. The endotoxin SEE, when presented to the class II-positive B cell lymphoblastoid line Raji, activates the V β 8-expressing T cell line Jurkat to secrete IL-2 (Herman et al, 1990, J. Exp. Med. 172:709). Since Jurkat cells express high level of CD28 and Raji cells express CD80/86, this reaction is partially dependant on CD28. We measured synthesis of interleukin-2 in this assay by ELISA (ELISA Max™ Set Deluxe Human IL-2 Kit; Biolegend #431805) after 48h, in the presence of increasing concentrations of hVH/VL CD28.3-short γ 1 hinge- γ 4CH2-CH3 domains.

[0083] The results are shown on Figure 9. They reveal that hCD28.3 monovalent antibodies reduce IL-2 synthesis by T cells in a dose-dependent manner.

EXAMPLE 7: PREPARATION OF A PEGYLATED hCD28.3 MONOVALENT ANTIBODY

[0084] A hCD28.3 Fab fragment prepared as described in Example 1 was pegylated with maleimide-activated 40KDa PEG using standard conditions for reduction and PEGylation.

[0085] Briefly, Fab antibody fragments were concentrated to 1 mg/mL and then diafiltrated against 20 mM Sodium phosphate, 2 mM EDTA, and pH 7.0. Fab' antibody fragments were then reduced by addition of cysteamine chloride in a molar equivalent ratio = 30:1 at room temperature. After 5 hours, solution was applied on a desalting column. Polyethylene glycol (PEG) (Sunbright GL2 400MA, NOF Corporation) was dissolved in 20 mM Phosphate, 2 mM EDTA, pH 7.0 to give 9% (w/w) solution. Desalted Fab solution and PEG were mixed in a molar equivalent ratio = 1:1.5 and incubated at ambient temperature for 3 h. Following PEGylation the Fab-peg was purified by chromatography using SP Sepharose HP medium. The target protein was eluted with a salt gradient from 0 to 1 M NaCl. Eluted peaks were analysed by SDS-Page. Peak 1 represented monopegylated material, peak 2 unpegylated material and peak 3 polypegylated material.

[0086] The results are shown on Figure 10.

[0087] These results show that a significant part of the Fab proteins from the CD28.3 mAb presents a perturbed pegylation profile which results in a yield of monopegylated Fabs of about 5% only (peak 1).

[0088] The CD28.3 mAb contains a cystein residue (C96) that is not engaged in intra or inter-chain disulfide bridges, at position 96 of the variable domain light chain. Free cystein will possess a higher reactivity than cystein residues engaged into disulfide bridges and will therefore preferentially be targets of maleimide-activated pegs. Therefore it is likely that a second, unwanted pegylation occurs on this residue.

[0089] To solve that problem we performed a VL- C96 mutation study to determine whether it was possible to substitute the C96 residue by another amino acid without modifying the binding properties of the antibody.

[0090] Plasmid coding for humanized anti-CD28.3 Fabs with unmodified C96 in the light chain, or with C96 to A, G, S, V, T, N or R mutations were constructed and transfected into Cos cells by lipofection, as disclosed in Example 1. Cell supernatants were first analyzed by sandwich ELISA to determine total Fab concentration, as disclosed in Example 2. Then supernatants were analysed by ELISA to determine binding activity on immobilized recombinant CD28, as disclosed in Example 2.

[0091] The results are shown in Figure 11. These results show that unlike all other substitutions tested, the C96A substitution resulted in a fully active antibody and that the C96N substitution resulted only in a moderate reduction of activity.

[0092] The C96A Fab fragment variant was pegylated and purified by chromatography as described above. Pegylated proteins pre-chromatography and elution peaks were analysed by

SDS-Page. The results are shown on Figure 12. Peak 1 represents monopegylated material.

[0093] These results show that the C96A Fab fragment can be pegylated with an efficacy reaching 41% (Figure 12).

[0094] Advantage of the CAA C-terminal end of the heavy chain. The immediate molecular environment of a free cysteine might modify its accessibility to maleimide-pegylation and therefore modify the yield of the pegylation reaction. One possible option for the C-terminal cysteine is to be the last amino acid of the heavy chain. Another option is the addition of "stuff amino acids" at the C-terminal position, after the last cysteine. We therefore compared pegylation efficacy of a Fab' molecule from the C96A variant of the humanized CD28.3 Mab with the last C-terminal cysteine being the last amino acid of the heavy chain (C variant; data shown in Figure 12) with a similar molecule with the last C-terminal cysteine being followed by two alanines (CAA variant). Our data clearly and reproducibly demonstrated that the CAA variant could be pegylated with a 20% higher efficacy (Figure 13). Indeed pegylation yield that was of 41% for the C96A-C variant reached 52% for the C96A-CAA variant.

SEQUENCE LISTING

[0095]

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 INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE
 (INSERM)
 MARY, Caroline
 POIRIER, Nicolas
 VANHOVE, Bernard

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Val His Ser Lys Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Lys
20          25          30

Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr
35          40          45

Phe Thr Glu Tyr Ile Ile His Trp Ile Lys Leu Arg Ser Gly Gln Gly
50          55          60

Leu Glu Trp Ile Gly Trp Phe Tyr Pro Gly Ser Asn Asp Ile Gln Tyr
65          70          75          80

Asn Ala Gln Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser
85          90          95

Ser Thr Val Tyr Met Glu Leu Thr Gly Leu Thr Pro Glu Asp Ser Ala
100         105         110

Val Tyr Phe Cys Ala Arg Arg Asp Asp Phe Ser Gly Tyr Asp Ala Leu
115         120         125

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Pro Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala Asp Lys Thr
130                      135                      140

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser
145                      150                      155                      160

Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
165                      170                      175

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro
180                      185                      190

Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
195                      200                      205

Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val
210                      215                      220

Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
225                      230                      235                      240

Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr
245                      250                      255

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
260                      265                      270

Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys
275                      280                      285

Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
290                      295                      300

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
305                      310                      315                      320

Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser
325                      330                      335

Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
340                      345                      350

Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
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<210> 11

<211> 1068

<212> DNA

<213> Artificial Sequence

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<223> hVLCD28.3-short hinge-gamma4CH2CH3

<400> 11

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atcacctgta agaccaacga gaacatctac tccaacctgg cctgggtatca gcagaaggac      180
ggcaagtccc ctcaagtctg gatctacgcc gccacccatc tggaggaggg cgtgccctct      240
agattctccg gctccggctc tggcaccag tactccctga ccacagctc cctgcagcct      300
gaggacttcg gcaactacta ctgccagcac ttctggggca ccccttgtag cttcggcgga      360
ggcaccaagc tggaaatcaa gcgggacaaa actcacacat gccaccctg cccagcacct      420
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atctcccgga cccctgaggt cacgtgcgtg gtggaggagc tgagccagga agaccccgag      540
gtccagttca actggtacgt ggatggcgtg gaggtgcata atgccaagac aaagccgcgg      600
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<213> Artificial Sequence

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20          25          30

Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Thr Asn Glu Asn
35          40          45

Ile Tyr Ser Asn Leu Ala Trp Tyr Gln Gln Lys Asp Gly Lys Ser Pro
50          55          60

Gln Leu Leu Ile Tyr Ala Ala Thr His Leu Val Glu Gly Val Pro Ser
65          70          75          80

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Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu Thr Ile Ser
85 90 95

Ser Leu Gln Pro Glu Asp Phe Gly Asn Tyr Tyr Cys Gln His Phe Trp
100 105 110

Gly Thr Pro Cys Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
115 120 125

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly
130 135 140

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
145 150 155 160

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln
165 170 175

Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val
180 185 190

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr
195 200 205

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
210 215 220

Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile
225 230 235 240

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
245 250 255

Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser
260 265 270

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
275 280 285

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
290 295 300

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val
305 310 315 320

Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met
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Leu Gly Lys
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<210> 13

<211> 1122

<212> DNA

<213> Artificial Sequence

<220>

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<400> 13

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tcctgcgaagg cctccggcta caccttcacc gagtacatca tccactggat caagctgaga      180
tccggccagg gcctggaatg gatcggtggt ttctaccctg gctccaacga catccagtac      240
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atggaactga ccggcctgac ccctgaggac tccgccgtgt acttctgcgc caggcgggac      360
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ctgggggggac catcagtcct cctgttcccc ccaaaaccca aggacactct catgatctcc      540
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caggaggaga tgaccaagaa ccaggtcagc ctgacctgcc tggtaaagg cttctacccc      900
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cctcccgtgc tggactccga cggctccttc ttctctaca gcaggctcac cgtggacaag      1020
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<210> 14

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1           5           10          15
Val His Ser Lys Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Lys
20          25          30
Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr

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35	40	45
Phe Thr Glu Tyr Ile Ile His Trp Ile Lys Leu Arg Ser Gly Gln Gly		
50	55	60
Leu Glu Trp Ile Gly Trp Phe Tyr Pro Gly Ser Asn Asp Ile Gln Tyr		
65	70	75
Asn Ala Gln Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser		
	85	90
Ser Thr Val Tyr Met Glu Leu Thr Gly Leu Thr Pro Glu Asp Ser Ala		
	100	105
Val Tyr Phe Cys Ala Arg Arg Asp Asp Phe Ser Gly Tyr Asp Ala Leu		
	115	120
Pro Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala Glu Pro Lys		
	130	135
Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Phe		
	145	150
Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr		
	165	170
Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val		
	180	185
Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val		
	195	200
Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser		
	210	215
Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu		
	225	230
Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser		
	245	250
Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro		
	260	265
Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln		
	275	280
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala		
	290	295
Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr		
	305	310
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu		
	325	330
Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser		
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<210> 15

<211> 1083

<212> DNA

<213> Artificial Sequence

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gagaacaact acaagaccac gcctcccggt ctggactccg acggctcctt cttcctctac	960
agcaggctca ccgtggacaa gagcagggtg caggagggga atgtcttctc atgctccgtg	1020
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tga	1083

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<211> 360

<212> PRT

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20 25 30

Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Thr Asn Glu Asn
35 40 45

Ile Tyr Ser Asn Leu Ala Trp Tyr Gln Gln Lys Asp Gly Lys Ser Pro
50 55 60

Gln Leu Leu Ile Tyr Ala Ala Thr His Leu Val Glu Gly Val Pro Ser
65 70 75 80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu Thr Ile Ser
85 90 95

Ser Leu Gln Pro Glu Asp Phe Gly Asn Tyr Tyr Cys Gln His Phe Trp
100 105 110

Gly Thr Pro Cys Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
115 120 125

Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
130 135 140

Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
145 150 155 160

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
165 170 175

Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val
180 185 190

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
195 200 205

Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
210 215 220

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly
225 230 235 240

Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
245 250 255

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr
260 265 270

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
275 280 285

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
 290 295 300

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
 305 310 315 320

Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe
 325 330 335

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
 340 345 350

Ser Leu Ser Leu Ser Leu Gly Lys
 355 360

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Anti-CD28-antistof med et CD28-bindingssted bestående af:

- et første variabelt domæne (også defineret heri som det "tung kæde variable domæne") defineret af følgende sekvens:

5 VQLQQSGAELKKPGASVKVSCKASGYTFTEYIIHWIKLRSGQGLEWIGWFYP
GSNDIQYNAQFKGKATLTADKSSSTVYMELTGLTPEDSAVYFCARRDDFSGY
DALPYWGQGTLVTVSA (SEQ ID NO:1), hvor det variable domæne
yderligere omfatter en Q-rest ved dens N-terminale ende;

- et andet variabelt domæne (også defineret heri som det "let kæde variable domæne") defineret af følgende sekvens:

10 DIQMTQSPSSLSASVGDRVTITCKTNENIYSNLAWYQQKDGKSPQLLIYAATH
LVEGVPSRFSGSGSGTQYSLTISSLQPEDFGNYQCQHFWGTPXTFGGGTKLEI KR,
hvor X = C eller A.

15 **2.** Antistof ifølge krav 1, der er et monovalent antistof.

3. Monovalent antistof ifølge krav 2, der er en heterodimer af:

- en første proteinkæde med sekvensen af aminosyrerne 21-251 af SEQ ID NO:4

20 - en anden proteinkæde med sekvensen af aminosyrerne 21-234 af SEQ ID NO:6.

4. Monovalent antistof ifølge krav 3, hvor den anden proteinkæde omfatter et variabelt domæne af SEQ ID NO: 2 hvor X betegner en alanin-rest.

25

5. Monovalent antistof ifølge krav 4, der er pegyleret.

6. Polynukleotid der koder for et antistof ifølge et hvilket som helst af kravene 1 til 4.

30

7. Terapeutisk sammensætning der omfatter et antistof ifølge et hvilket som helst af kravene 1 til 5.

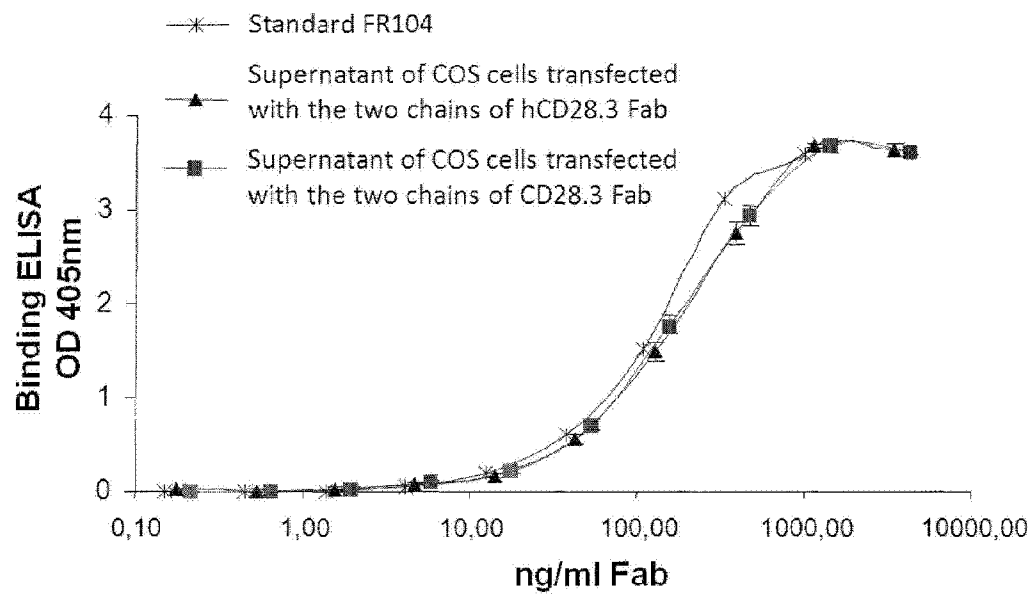
8. Antistof ifølge et hvilket som helst af kravene 1 til 5, til anvendelse i behandling
5 af en patologisk tilstand valgt fra gruppen bestående af transplantatafstødning, kronisk allotransplantat-vaskulopati, graft-versus-host sygdom, T-lymfocyt-medierede autoimmune sygdomme, hypertension, allergiske fænomener og kronisk inflammatoriske sygdomme.

DRAWINGS

9
 ATG GAA TGG TGC TTT CTC TTC TTT CTC CTC TCA ACT GCA GGT GTC CAC TCC AAG GTC V Q L Q Q S G A
 M E W C W V F L F L L L S V T A G V H S K V Q L Q Q S G A
 37
 GAG CTG AAG AAA CCC GGG GCG TCG GTG AAA GTC TCC TCG AAG GCG TCT GGT TAC ACC TTC ACT GAA TAT ATT ATA CAC TGG ATA AAG
 E L K K P G A S V W W S C K A S G Y T F T E Y I I H W I K
 66
 CTG AAG TCT GGA CAG GGT CTT GAG TGG ATT GGG TGG TTT TAC CCT GGA AGT AAT GAT ATA CAG TAC AAT GCG CAA TTC AAG GGC AAG
 L R S G Q G L H W I G W F Y P G S N D I Q Y N A Q F K G K
 95
 GCG ACA TTG ACT GCG GAC AAA TCC TCC AGC ACC GTC TAT ATG GAA CTT ACT GGA TTG ACA CCC GAG GAC TCT GCG GTC TAT TTT TGT
 A T L I A D K S S S T V Y M E L T G L T T E D S A V Y F C
 124
 GCA AGA CGC GAC GAT TTC TCI GGT TAC GAC GGC CTT CCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA GCT AGC ACC AAG
 A R R D D F S S G Y D A L P Y W C Q G T L V I V S A A S T K
 153
 GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC AAG AGC ACC TCT GGG GGC ACA GCG GGC CTG GGC TGC CTG GTC AAG GAC TAC TTC
 G P S V F P L A P S S S K S T S G G T A A L G C L V K D Y F
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 CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GGC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC
 P F P V T V S W N S G A L T S G V H T F P A V L Q S S G L
 211
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 Y S L S V V T V P S S S L G T Q F Y I C N V N H K P S N
 231
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Figure 1

A



B

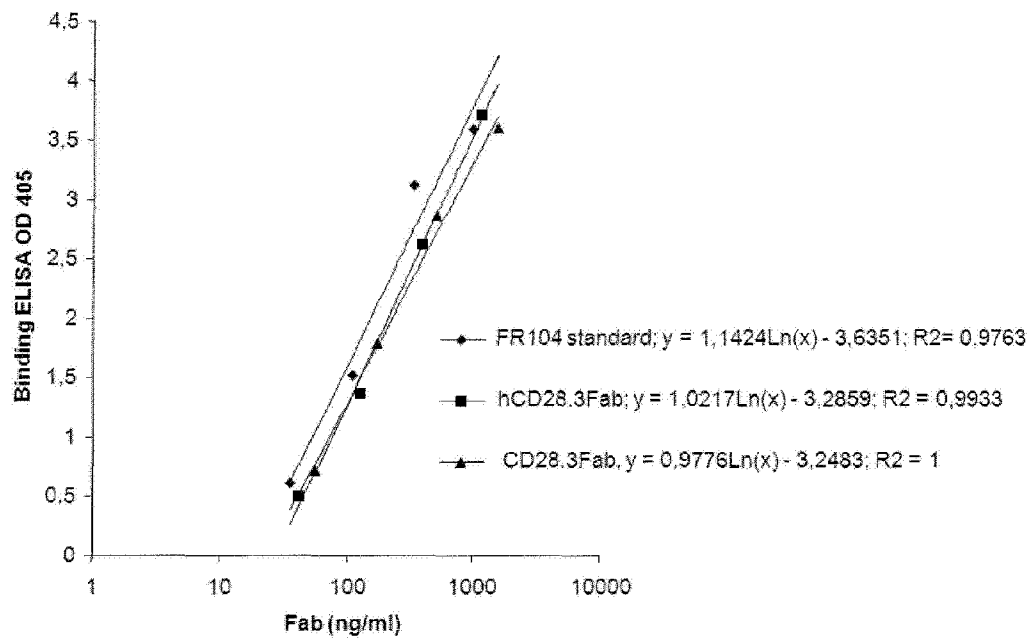


Figure 3

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46	GGC GTG CAC TCC AAG CAG GTG CAG CTG CAG CAG TCT GGC GCC GAG	90
16	Gly Val His Ser Lys Gln Val Gln Leu Gln Ser Gly Ala Glu	30
91	CTG AAG AAG CCT GGC GCC TCC GTC AAG GTG TCC TGC AAG GCC TCC	135
31	Leu Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser	45
136	GGC TAC ACC TTC ACC GAG TAC ATC ATG CAC TGG ATC AAG CTG AGA	180
46	Gly Tyr Thr Phe Thr Glu Tyr Ile Ile His Trp Ile Lys Leu Arg	60
181	TCC GGC CAG GGC CTG GAA TGG ATC GGC TGG TTC TAC CCT GGC TCC	225
61	Ser Gly Gln Gly Leu Glu Trp Ile Gly Trp Phe Tyr Pro Gly Ser	75
226	AAC GAC ATC CAG TAC AAC GCC CAG TTC AAG GGC AAG GCC ACC CTG	270
76	Asn Asp Ile Gln Tyr Asn Ala Gln Phe Lys Gly Lys Ala Thr Leu	90
271	ACC GCC GAC AAG TCC TCC TCC ACC GTG TAC ATG GAA CTG ACC GGC	315
91	Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr Met Glu Leu Thr Gly	105
316	CTG ACC CGT GAG GAC TCC GCC GTG TAC TTC TGC GCC AGG CCG GAC	360
106	Leu Thr Pro Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Arg Asp	120
361	CAC TTC TCT GGC TAC GAC GCC CTG CCT TAT TGG GCC CAG GCC ACC	405
121	Asp Phe Ser Gly Tyr Asp Ala Leu Pro Tyr Trp Gly Gln Gly Thr	135
406	CTG GTG ACC GTG TCC GCC GAC AAA ACT CAC ACA TGC CCA CCG TGC	450
136	Leu Val Thr Val Ser Ala Asp Lys Thr His Thr Cys Pro Pro Cys	150
451	CCA GCA COT GAG TTC CTG GGC GGA CCA TCA GTC TTC CTG TTC CCC	495
151	Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro	165
496	CCA AAA CCC AAG GAC ACT CTC ATG ATC TCC CCG ACC CCT GAG GTC	540
166	Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val	180
541	ACG TGC GTG GTG GTG GAC GTG AGC CAG GAA GAC CCC GAG GTC CAG	585
181	Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln	195
586	TTC AAC TGG TAC GTG GAT GGC GTG GAG GTG CAT AAT GCC AAG ACA	630
196	Phe Asn Trp Tyr Val Asp Gly Val His Asn Ala Lys Thr	210
631	AAG CCG CCG GAG GAG CAG TTC AAC AGC AGC TAC CGT GTG GTC AGC	675
211	Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser	225
676	GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAC GGC AAG GAG TAC	720
226	Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr	240
721	AAG TGC AAG GTC TCC AAC AAA GGC CTC CCC TCC TCC ATC GAG AAA	765
241	Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys	255
766	ACC ATC TCC AAA GCC AAA GGC CAG CCC CGA GAG CCA CAG GTC TAC	810
256	Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr	270
811	ACC CTG CCC CCA TCC CAG GAG GAG ATG ACC AAG AAC CAG GTC AGC	855
271	Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser	285
856	CTG ACC TGG CTG GTC AAA GGC TTC TAC CCC AGC CAG ATC GCC GTG	900
286	Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val	300
901	GAG TGG GAG AGC AAT GGC CAG CCG GAG AAC AAC TAC AAG ACC AGG	945
301	Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr	315
946	CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AGG	990
316	Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg	330
991	CTC ACC GTG CAC AAG AGC AGC TGG CAG GAG GGC AAT GTC TTC TCA	1035
331	Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser	345
1036	TGC TCC GTG ATG CAT GAG GGT CTG CAC AAC CAC TAC ACA CAG AAG	1080
346	Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys	360
1081	AGC CTC TCC CTG TCT CTG GGT AAA TGA	1107
361	Ser Leu Ser Leu Ser Leu Gly Lys End	

Figure 4

1	ATG TCC GTG CCT ACC CAG GTC CTG GGA CTG CTG CTG CTG TGG CTG	45
1	Met Ser Val Pro Thr Gln Val Leu Gly Leu Leu Leu Leu Trp Leu	15
46	ACC GAC GCC AGA TGC GAC ATC CAG ATG ACC CAG TCC CCC TCC TCC	90
16	Thr Asp Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser	30
91	CTG TCT GCC TCC GTG GGC GAC CGG GTG ACC ATC ACC TGT AAG ACC	135
31	Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Thr	45
136	AAC GAG AAC ATC TAC TCC AAC CTG GCC TGG TAT CAG CAG AAG GAC	180
46	Asn Glu Asn Ile Tyr Ser Asn Leu Ala Trp Tyr Gln Gln Lys Asp	60
181	GGC AAG TCC CCT CAG CTG CTG ATC TAC GCC GCC ACC CAT CTG GTG	225
61	Gly Lys Ser Pro Gln Leu Leu Ile Tyr Ala Ala Thr His Leu Val	75
226	GAG GGC GTG CCC TCT AGA TTC TCC GGC TCC GGC TCT GGC ACC CAG	270
76	Glu Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln	90
271	TAC TCC CTG ACC ATC AGC TCC CTG CAG CCT GAG GAC TTC GGC AAC	315
91	Tyr Ser Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Gly Asn	105
316	TAC TAC TGC CAG CAC TTC TGG GGC ACC CCT TGT ACC TTC GGC GGA	360
106	Tyr Tyr Cys Gln His Phe Trp Gly Thr Pro Cys Thr Phe Gly Gly	120
361	GGC ACC AAG CTG GAA ATC AAG CGG GAC AAA ACT CAC ACA TGC CCA	405
121	Gly Thr Lys Leu Glu Ile Lys Arg Asp Lys Thr His Thr Cys Pro	135
406	CCG TGC CCA GCA CCT GAG TTC CTG GGG GGA CCA TCA GTC TTC CTG	450
136	Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu	150
451	TTC CCC CCA AAA CCC AAG GAC ACT CTC ATG ATC TCC CGG ACC CCT	495
151	Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro	165
496	GAG GTC ACG TGC GTG GTG GTG GAC GTG AGC CAG GAA GAC CCC GAG	540
166	Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu	180
541	GTC CAG TTC AAC TGG TAC GTG GAT GGC GTG GAG GTG CAT AAT GCC	585
181	Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala	195
586	AAG ACA AAG CCG CGG GAG GAG CAG TTC AAC AGC ACG TAC CGT GTG	630
196	Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val	210
631	GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAC GGC AAG	675
211	Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys	225
676	GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC CTC CCG TCC TCC ATC	720
226	Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile	240
721	GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAG CCA CAG	765
241	Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln	255
766	GTC TAC ACC CTG CCC CCA TCC CAG GAG GAG ATG ACC AAG AAC CAG	810
256	Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln	270
811	GTC AGC CTG ACC TCC CTG GTC AAA GGC TTC TAC CCC AGC GAC ATC	855
271	Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile	285
856	GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG	900
286	Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys	300
931	ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC	945
331	Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr	315
946	AGC AGG CTC ACC GTG GAC AAG AGC AGG TGG CAG GAG GGG AAT GTC	990
316	Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val	330
991	TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACA	1035
331	Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr	345
1036	CAG AAG AGC CTC TCC CTG TCT CTG GGT AAA TGA	1068
346	Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys End	

Figure 5

1	ATG GAA TCG TGC TGG GTG TTC CTG TTC CTG CTG TCC GTG ACC GCT	45
1	Met Glu Trp Cys Trp Val Phe Leu Phe Leu Leu Ser Val Thr Ala	15
46	GGC GTG CAC TCC AAG CAG GTG CAG CTG CAG CAG TCT GGC GCC GAG	90
16	Gly Val His Ser Lys Gln Val Gln Leu Gln Gln Ser Gly Ala Glu	30
91	CTG AAG AAG CCT GGC GCC TCC GTC AAG GTG TCC TGC AAG GCC TCC	135
31	Leu Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser	45
136	GGC TAC ACC TTC ACC GAG TAC ATC ATC CAC TGG ATC AAG CTG AGA	180
46	Gly Tyr Thr Phe <u>Thr Gln Tyr Ile</u> Ile His Trp Ile Lys Leu Arg	60
181	TCC GGC CAG GGC CTG GAA TGG ATC GGC TGG TTC TAC CCT GGC TCC	225
61	Ser Gly Gln Gly Leu Glu Trp <u>Ile Gly Trp Phe Tyr Pro Gly Ser</u>	75
226	AAC GAC ATC CAG TAC AAC GCC CAG TTC AAG GGC AAG GCC ACC CTG	270
76	<u>Asn Asp Ile Gln Tyr Asn Ala Gln Phe</u> Lys Gly Lys Ala Thr Leu	90
271	ACC GCC GAC AAG TCC TCC TCC ACC GTG TAC ATG GAA CTG ACC GGC	315
91	Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr Met Glu Leu Thr Gly	105
316	CTG ACC CCT GAG GAC TCC GCC GTC TAC TTC TGC GCC AGG CCG GAC	360
106	Leu Thr Pro Glu Asp Ser Ala Val Tyr Phe Cys <u>Ala Arg Arg Asp</u>	120
361	GAC TTC TCT GGC TAC GAC GCC CTG CCT TAT TGG GGC CAG GGC ACC	405
121	<u>Asp Phe Ser Gly Tyr Asp Ala Leu Pro Tyr Trp Gly Gln Gly Thr</u>	135
406	CTG GTG ACC GTG TCC GCC GAG CCC AAA TCT TGT GAC AAA ACT CAC	450
136	Leu Val Thr Val Ser Ala <u>Glu Pro Lys Ser Cys Asp Lys Thr His</u>	150
451	ACA TGC CCA CCG TGC CCA GCA CCT GAG TTC CTG GGG GGA CCA TCA	495
151	<u>Thr Cys Pro Pro Cys Pro</u> Ala Pro Glu Phe Leu Gly Gly Pro Ser	165
496	GTC TTC CTG TTC CCC CCA AAA CCC AAG GAC ACT CTC ATG ATC TCC	540
166	<u>Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser</u>	180
541	CGG ACC CCT GAG GTC ACG TGC GTG GTG GTG GAC GTG AGC CAG GAA	585
181	<u>Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu</u>	195
586	GAC CCC GAG GTC CAG TTC AAC TGG TAC GTG GAT GGC GTG GAG GTG	630
196	<u>Asp Pro Gln Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val</u>	210
631	CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTC AAC AGC ACG	675
211	<u>His Asn Ala Lys Thr Lys Pro Arg Glu Glu Glu Phe Asn Ser Thr</u>	225
676	TAC CGT GTG GTC ACC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG	720
226	<u>Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu</u>	240
721	AAC GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC CTC CCG	765
241	<u>Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro</u>	255
766	TCC TCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA	810
256	<u>Ser Ser Ile Gln Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg</u>	270
811	GAG CCA CAG GTG TAC ACC CTG CCC CCA TCC CAG GAG GAG ATG ACC	855
271	<u>Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr</u>	285
856	AAG AAC CAC CTC ACC CTG ACC TGC CTC CTC AAA GGC TTC TAC CCC	900
286	<u>Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro</u>	300
901	AGC GAC ATC CCC GTG GAG TGG GAG AGC AAT GGG CAC CCG CAC AAC	945
301	<u>Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn</u>	315
946	AAC TAC AAG ACC ACG CCT CCC CTG CTG GAC TCC CAC GGC TCC TTC	990
316	<u>Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe</u>	330
991	TTC CTC TAC AGC AGC CTC ACC GTG GAC AAG AGC AGG TGG CAG GAG	1035
331	<u>Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu</u>	345
1036	GGG AAT GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC	1080
346	<u>Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn</u>	360
1081	CAC TAC ACA CAG AAG AGC CTC TCC CTG TCT CTG GGT AAA TGA	1122
361	<u>His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys End</u>	

Figure 6

1	ATG TCC GTG CCT ACC CAG GTG CTG GGA CTG CTG CTG CTG TGG CTG	45
1	Met Ser Val Pro Thr Gln Val Leu Gly Leu Leu Leu Leu Trp Leu	15
46	ACC GAC GCC AGA TGC GAC ATC CAG ATG ACC CAG TCC CCC TCC TCC	90
16	Thr Asp Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser	30
91	CTG TCT GCC TCC GTG GGC GAC CGG GTG ACC ATC ACC TGT AAG ACC	135
31	Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Thr	45
136	AAC GAG AAC ATC TAC TCC AAC CTG GCC TGG TAT CAG CAG AAG GAC	180
46	Asn Glu Asn Ile Tyr Ser Asn Leu Ala Trp Tyr Gln Gln Lys Asp	60
181	GGC AAG TCC CCT CAG CTG CTG ATC TAC GCC GCC ACC CAT CTG GTG	225
61	Gly Lys Ser Pro Gln Leu Leu Ile Tyr Ala Ala Thr His Leu Val	75
226	GAG GGC GTG CCC TCT AGA TTC TCC GGC CCC GGC TCT GGC ACC CAG	270
76	Glu Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln	90
271	TAC TCC CTG ACC ATC AGC TCC CTG CAG CCT GAG GAC TTC GGC AAC	315
91	Tyr Ser Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Gly Asn	105
316	TAC TAC TGC CAG CAC TTC TGG GGC ACC CCT TGT ACC TTC GGC GGA	360
106	Tyr Tyr Cys Gln His Phe Trp Gly Thr Pro Cys Thr Phe Gly Gly	120
361	GGC ACC AAG CTG GAA ATC AAC CCG GAG CCC AAA TCT TGT GAC AAA	405
121	Gly Thr Lys Leu Glu Ile Lys Arg Glu Pro Lys Ser Cys Asp Lys	135
406	ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAG TTC CTG GGC GGA	450
136	Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly	150
451	CCG TCA GTC TTC CTG TTC CCC CCA AAA CCC AAG CAC ACT CTC ATG	495
151	Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met	165
496	ATC TCC CGG ACC CCT GAG GTC ACG TGC GTG GTG GTG GAC GTG AGC	540
166	Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser	180
541	CAG GAA GAC CCC GAG GTC CAG TTC AAC TGG TAC GTG GAT GGC GTG	585
181	Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val	195
586	GAG GTG CAT AAT GCC AAG ACA AAG CCG CCG GAG GAG CAG TTC AAC	630
196	Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn	210
631	AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC	675
211	Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp	225
676	TGG CTG AAC GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC	720
226	Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly	240
721	CTC CCG TCC TCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGC CAG	765
241	Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln	255
766	CCC CCA GAG CCA CAG GTG TAC ACC CTG CCC CCA TCC CAG GAG GAG	810
256	Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu	270
811	ATG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC CTC	855
271	Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe	285
856	TAC CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG	900
286	Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro	300
901	GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC	945
301	Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly	315
946	TCC TTC TTC CTC TAC AGC AGG CTC ACC GTG GAC AAG AGC AGG TGG	990
316	Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg Trp	330
991	CAG GAG GGG AAT GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG	1035
331	Glu Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu	345
1036	CAC AAC CAC TAC ACA CAG AAG AGC CTC TCC CTG TCT CTG GGT AAA	1080
346	His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys	360
1081	TGA	1083
361	End	

Figure 7

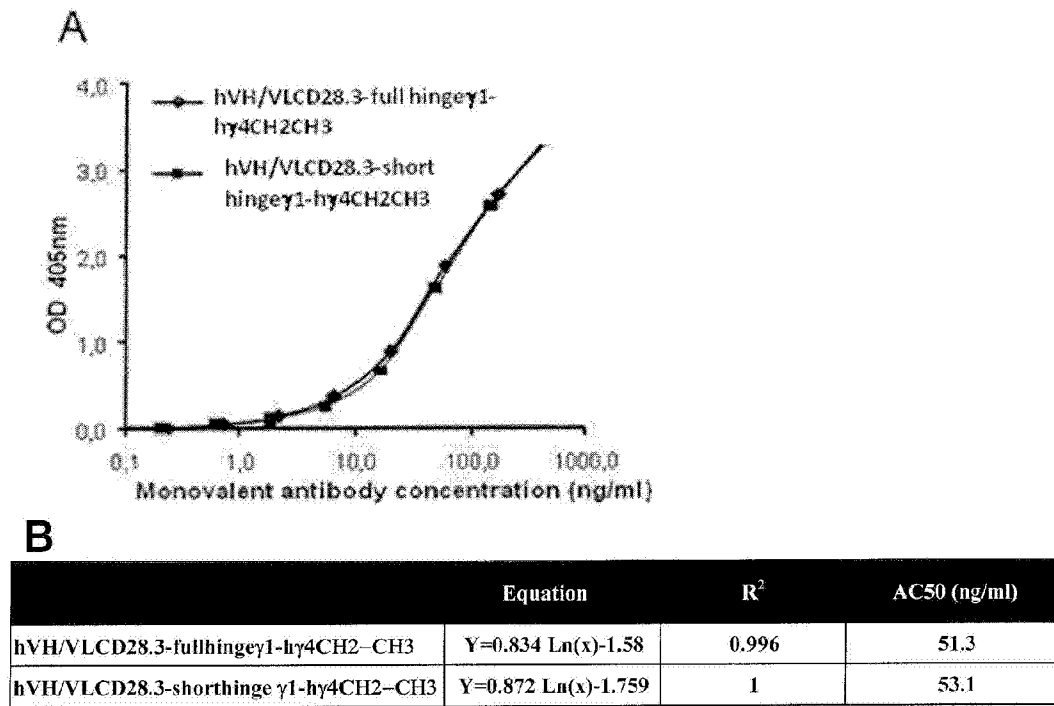


Figure 8

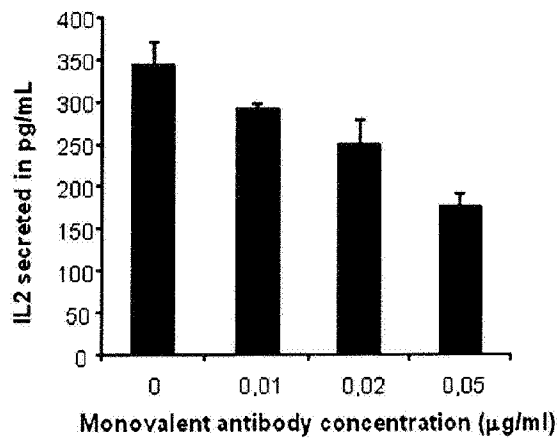


Figure 9

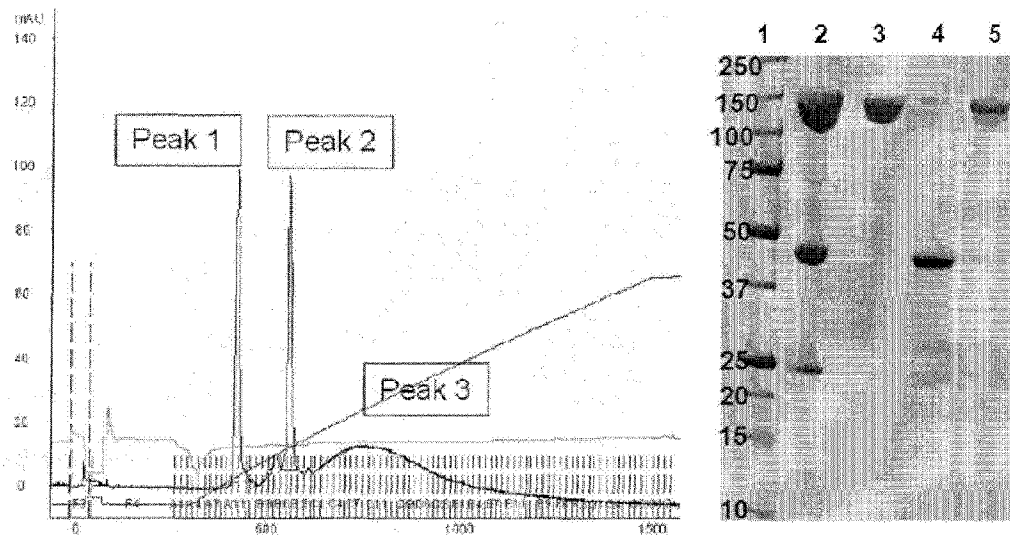


Figure 10

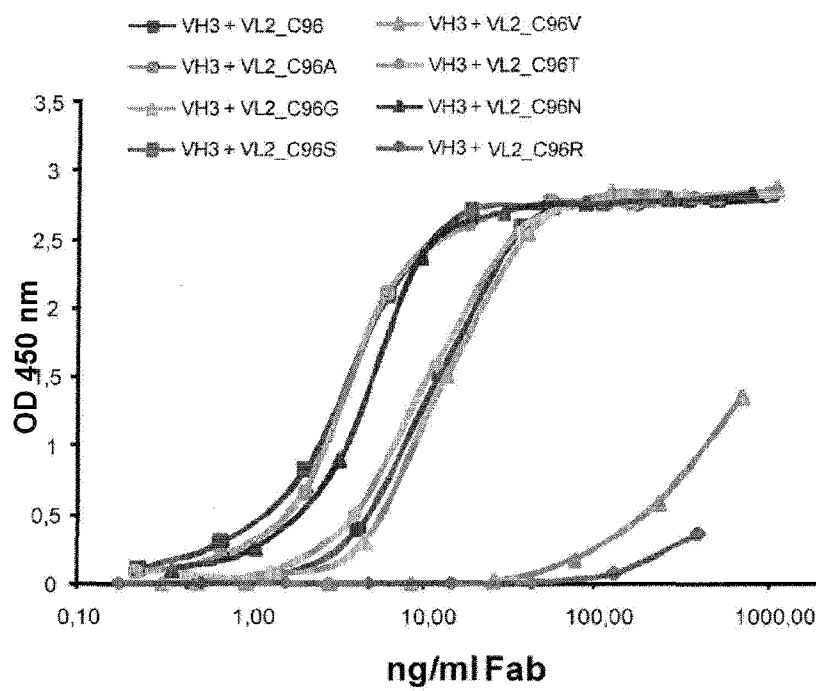


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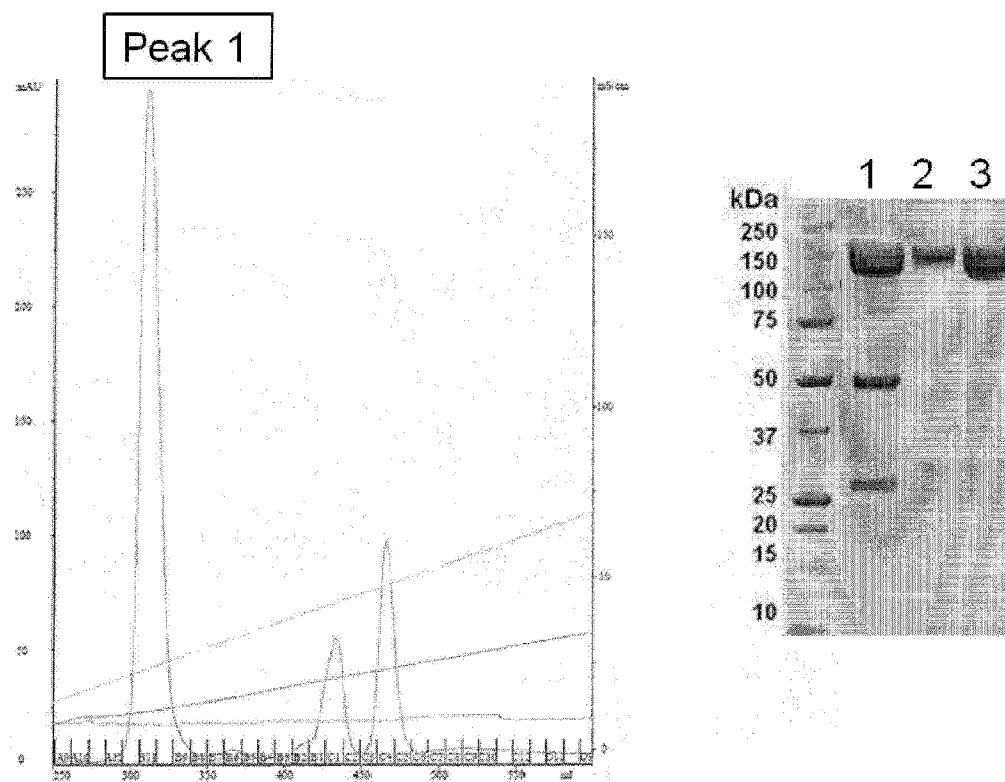
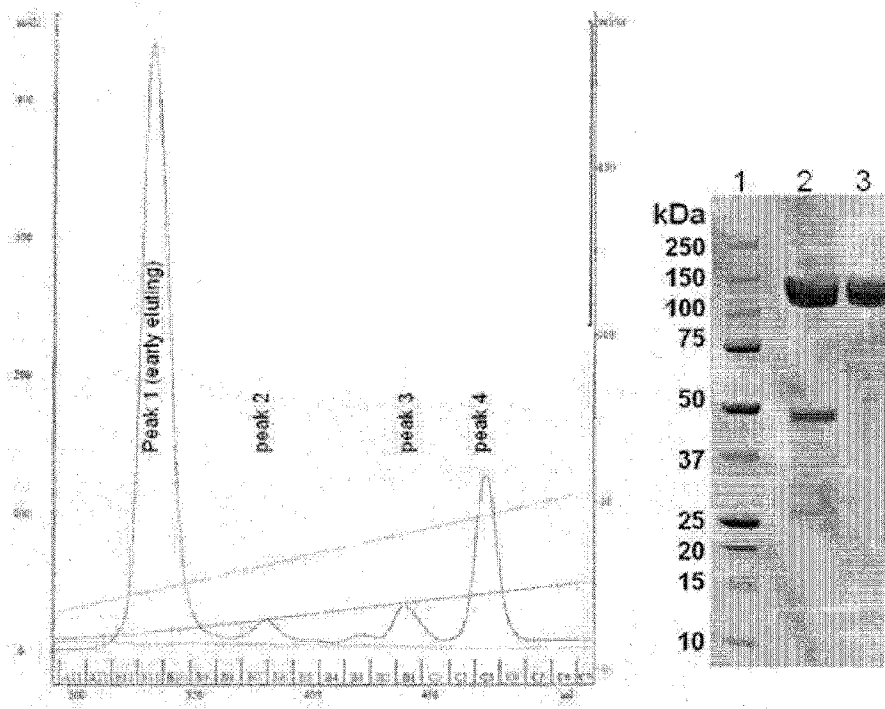


Figure 12

**Figure 13**