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(54) Title: METHODS FOR CONTROLLING FUCOSYLATION LEVELS IN PROTEINS

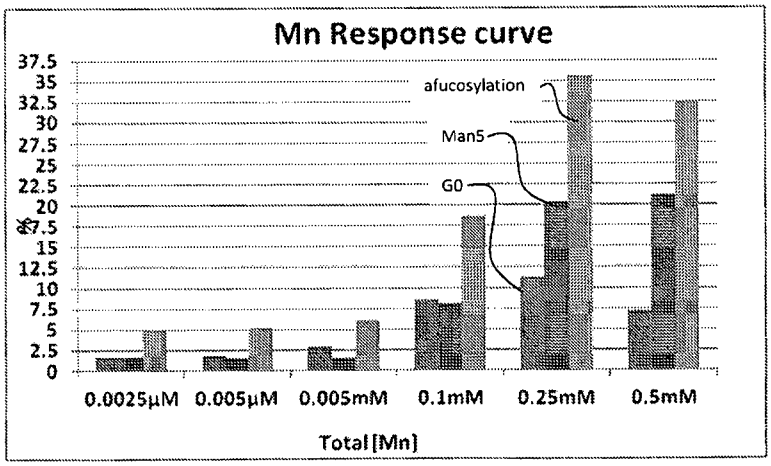

Fig. 12

(57) Abstract: The present invention relates to a method or process for controlling, inhibiting or reducing protein fucosylation in a eukaryote and/or a eukaryotic protein expression system. Said method comprises carrying out the protein expression and/or post-translational modification in the presence of an elevated total concentration of manganese or manganese ions.

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METHODS FOR CONTROLLING FUCOSYLATION LEVELS IN PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of and the priority to provisional Indian Patent applications 3262/CHE/2013 filed on 23 July 2013 and 3265/CHE/2013 filed on 23 July 2013 with the Indian Patent Office. The content of the said applications filed on 23 July 2013 is incorporated herein by reference for all purposes in its entirety, including an incorporation of any element or part of the decision, claims or drawings not contained herein and referred to in Rule 20.5(a) of PCT, pursuant to Rule 4.18 of the PCT.

TECHNICAL FIELD

The present invention relates to methods for controlling fucosylation levels in proteins.

BACKGROUND OF THE DISCLOSURE

Proteins expressed in eukaryotic expression systems undergo a process of post-translational modification, which involves glycosylation. Eukaryotic expression systems which have been established today for the production of glycoproteins, like IgG and other monoclonal antibodies comprising an Fc region add N-glycans to the polypeptide chains.

In IgG, the most important N-glycan is bound at Asn 297 of both CH2 chains (see Fig. 14), which comprises, among others, N-acetyl-neuraminic acid (sialic acid), N-acetyl-glucosamine, galactose, mannose, and fucose residues.

This applies, basically, for transgenic plant expression systems as well as for mammalian cell lines, insect cell lines etc. In all these cases, the N-glycan comprises at least one fucose residue which is bound either α-3-glycosidically or α-6-glycosidically to the N-acetyl-glucosamine residue bound to the Asn residue of the polypeptide chain.

Yeast expression systems tend to produce hyperglycoproteins rich in mannose, which often lead to unwanted immune reactions when the therapeutic antibody is administered to a patient. Baculovirus transfected insect cell systems cause problems due to hypoglycosylation, which negatively affects the effector function of therapeutic antibodies. Furthermore, the major disadvantage are the catalytitc properties of infectious baculovirus that narrows the window for full IgG production.

ADCC is a mechanism of cell-mediated immunity whereby an effector cell of the immune system actively lyses a target cell that has been bound by specific antibodies. It is one of the mechanisms through which antibodies, as part of the humoral immune response, can act to limit and contain infection. Classical ADCC-mediating effector cells are natural killer (NK) cells; but monocytes and eosinophils can also mediate ADCC. ADCC is part of the adaptive immune response due to its dependence on a prior antibody response.

Therapeutic antibodies which are used to elicit an ADCC in target cells need an Fc region in order to be recognized by Fc gamma receptors of the said effector cells.

Recent studies have shown that monoclonal antibodies having a reduced amount of fucose in its glycosylation pattern exhibit much higher Antibody-Dependent Cellular Cytotoxicity (ADCC) activity as compared to fucosylated antibodies. Again, it is basically position Asn 297 where a lack of fucose residues leads to the increased ADCC. The mechanism behind the increased ADCC of a low/no-fucose Antibody seems to be mediated by an increased affinity of a so

modified Fc region to FcγR, for example FcγIIIa (CD16), the major Fc receptor for ADCC in human immune effector cells (Shields et al, 2002).

Fucosylation is one of the most common modifications involving oligosaccharides on glycoproteins or glycolipids. Fucosylation comprises the attachment of a fucose residue to *N*-glycans, *O*-glycans, and glycolipids. *O*-Fucosylation, a special type of fucosylation, is very important for Notch signaling. The regulatory mechanisms for fucosylation are complicated. Many kinds of fucosyltransferases, the GDP-fucose synthesis pathway, and GDP-fucose transporter are involved in the regulation of fucosylation.

Glycosylation is known to impact the effector functions of therapeutic monoclonal antibodies. Among the various sugar residues in the oligosaccharide chain of an antibody, fucose is one of the key sugars that affects the antibody dependent cellular cytotoxicity (ADCC) induced by the product.

Manipulation of cell culture parameters is often employed to control galactosylation and sialylation of an antibody. Control of fucosylation is majorly done by using FUT8 knock out cells and other gene silencing models through cell line engineering.

US20090208500 discloses the production of antibodies with reduced fucose and improved Fc function by manipulation of FUT8 Knock out cells.

US7972810 discloses cell culturing methods and media containing manganese that improve glycosylation or sialylation of glycoproteins, including erythropoietin and analogs or derivatives thereof. According to the disclosure, manganese increases sialylation and site occupancy in case of O-linked and N-linked glycosylation (i.e. lower aglycosylated product) and also increases terminal galactosylation.

Further, fucose content of monoclonal antibodies can be controlled by culture medium osmolality for high antibody-dependent cellular cytotoxicity (Konno et al. 2012)

Yet, there is a need for an efficient method of producing glycoproteins in a desired cell line while controlling the fucose content of the recombinantly engineered antibodies without undergoing the laborious process of creating a FUT8 gene knockout in a selected cell line each time.

EMBODIMENTS OF THE INVENTION

These objects are met with methods and means according to the independent claims of the present invention. The dependent claims are related to preferred embodiments. It is yet to be understood that value ranges delimited by numerical values are to be understood to include the said delimiting values.

SUMMARY OF THE DISCLOSURE

Before the invention is described in detail, it is to be understood that this invention is not limited to the particular component parts of the devices described or process steps of the methods described as such devices and methods may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include singular and/or plural referents unless the context clearly dictates otherwise. It is moreover to be understood that, in case parameter ranges are given which are delimited by numeric values, the ranges are deemed to include these limitation values.

According to one embodiment of the invention, a method or process for modifying fucosylation in a eukaryote and/or a eukaryotic protein expression system is provided, in which method or process the total concentration of manganese or manganese ions in the medium is controlled.

Fucosylation of glycoproteins is accomplished by fucosyltransferases (FUT). These are enzymes that transfer an L-fucose sugar from a GDP-fucose (guanosine diphosphate-fucose) donor substrate to an acceptor substrate. The acceptor substrate can be another sugar such as the transfer of a fucose to a core GlcNAc (N-acetylglucosamine) sugar as in the case of N-linked glycosylation, or to a protein, as in the case of O-linked glycosylation produced by O-fucosyltransferase. There are various fucosyltransferases in mammals, the vast majority of which, are located in the Golgi apparatus. The O-fucosyltransferases have recently been shown to localize to the endoplasmic reticulum (ER). Examples of mammalian fucosyltransferases are FUT1; FUT2; FUT3; FUT4; FUT5; FUT6; FUT7; FUT8; FUT9; FUT10 and FUT11.

Manganese is an essential trace element which participates in many enzyme systems, although its role is not yet fully understood. It acts as a cofactor in enzymes that are essential for energy production and is involved in the metabolism of glucose, glycogen storage in the liver, protein digestion and synthesis of cholesterol and fatty acids. It is also essential for the synthesis of DNA and RNA molecules.

Manganese is essential for the growth and maintenance of the nervous system, the development and maintenance of bones and joints, the function of female sex hormones and thyroid hormones. Superoxide dismutase (SOD, MnSOD) is an antioxidant enzyme that in its structure contains manganese.

In extracellular liquids or Eukaryotes, manganese is practically absent, while in mammals, the intracellular concentration of Manganese is in the range of 0.010 picogram/cell – 0.10 picogram/cell.

However, the inventors surprisingly found that that the concentration of manganese has a direct effect on the fucosylation level of glycoproteins.

Thus, the present invention provides for modification of the fucose content of glycosylated proteins by varying the total concentration of manganese or manganese ions in media and feeds in the process.

Preferably, the method or process is a method or process to decrease fucosylation. In such method or process the protein expression and/or post-translational modification is carried out in the presence of an elevated total concentration of manganese or manganese ions.

Surprisingly, the inventors found that under such conditions, the glycoproteins expressed have a decreased fucosylation level. Further, they found that the cell growth, viability and the titre of the proteins produced is not effected by the elevation of manganese or manganese ion concentration.

Further, they found that other properties of the glycosylation pattern, namely G0 and Man5, are increased in the presence of an elevated total concentration of manganese or manganese ions.

As used herein, the term "fucosylation level" refers to the total amount of glycoproteins in which the glycans carry a fucose. Likewise, the terms "afucosylation level" and "% afucosylation" refers to the percentage of glycoprotein which have no fucose in their glycans.

In a preferred embodiment of the method or process according to the invention, it is provided that the elevated concentration of manganese or manganese ions is in the range of ≥ 0.05 mM - ≤ 10 mM.

Preferably, the elevated concentration of manganese or manganese ions is 0,05; 0,1; 0,15; 0,2; 0,25; 0,3; 0,35; 0,4; 0,45; 0,5; 0,55; 0,6; 0,65; 0,7; 0,75; 0,8; 0,85; 0,9; 0,95; 1; 1,05; 1,1; 1,15; 1,2; 1,25; 1,3; 1,35; 1,4; 1,45; 1,5; 1,55; 1,6; 1,65; 1,7; 1,75; 1,8; 1,85; 1,9; 1,95; 2; 2,05; 2,1; 2,15; 2,2; 2,25; 2,3; 2,35; 2,4; 2,45; 2,5; 2,55; 2,6; 2,65; 2,7; 2,75; 2,8; 2,85; 2,9; 2,95; 3; 3,05; 3,1; 3,15; 3,2; 3,25; 3,3; 3,35; 3,4; 3,45; 3,5; 3,55; 3,6; 3,65; 3,7; 3,75; 3,8; 3,85; 3,9; 3,95; 4; 4,05; 4,1; 4,15; 4,2; 4,25; 4,3; 4,35; 4,4; 4,45; 4,5; 4,55; 4,6; 4,65; 4,7; 4,75; 4,8; 4,85; 4,9; 4,95; 5; 5,05; 5,1; 5,15; 5,2; 5,25; 5,3; 5,35; 5,4; 5,45; 5,5; 5,55; 5,6; 5,65; 5,7; 5,75; 5,8; 5,85; 5,9; 5,95; 6; 6,05; 6,1; 6,15; 6,2; 6,25; 6,3; 6,35; 6,4; 6,45; 6,5; 6,55; 6,6; 6,65; 6,7; 6,75; 6,8; 6,85; 6,9; 6,95; 7; 7,05; 7,1; 7,15; 7,2; 7,25; 7,3; 7,35; 7,4; 7,45; 7,5; 7,55; 7,6; 7,65; 7,7; 7,75; 7,8; 7,85; 7,9; 7,95; 8; 8,05; 8,1; 8,15; 8,2; 8,25; 8,3; 8,35; 8,4; 8,45; 8,5; 8,55; 8,6; 8,65; 8,7; 8,75; 8,8; 8,85; 8,9; 8,95; 9; 9,05; 9,1; 9,15; 9,2; 9,25; 9,3; 9,35; 9,4; 9,45; 9,5; 9,55; 9,6; 9,65; 9,7; 9,75; 9,8; 9,85; 9,9; 9,95; or 10 mM.

These concentrations refer to the total concentration in the medium where the protein expression and/or post-translational modification takes place. This means that, e.g., feed solutions can have significantly higher concentration of manganese or manganese ions.

Preferably, the concentration of manganese is accomplished by adding manganese to the culture medium, and/or to the feed medium.

Likewise preferably, the manganese concentration is increased or decreased during protein expression and/or post-translational modification.

In a preferred embodiment of the method or process according to the invention, it is provided that the protein expression and/or post-translational modification is carried out in a protein expression system selected from the group consisting of

- Insect cells
- Fungal cells
- Yeast cells
- Protozoan cells, and/or
- Mammalian cells

Preferably, the mammalian cells are selected from the group consisting of murine cells (e.;g, NS0), hamster cells (e.g., CHO or BHK) and/or human cells (e.g., PER.C6).

Preferably, the protein is a glycoprotein. More preferably, the protein is a recombinant protein.

In a preferred embodiment of the method or process according to the invention, it is provided that the protein is an immunoligand.

The term "immunoligand" is used herein to mean an entity that has the capability to bind to another biological entity with a sufficient degree of sensitivity and/or specificity.

In another preferred embodiment of the method or process according to the invention, it is provided that immunologand is at least one selected from the group consisting of

- a monoclonal antibody (murine, chimeric, humanized, human), or derivative thereof
- a new antibody format
- a fusion peptide consisting of an immunoglobulin Fc region fused to a target binding moiety, e.g, a receptor fragment

The above listed immunoligands comprise, preferably, an Fc region or another domain that is capable of being glycosylated and/or binding to an Fc receptor, e.g., FcγRI (CD64), FcγRIIA (CD32), FcγRIIB (CD32), FcγRIIIA (CD16a), FcγRIIIB (CD16b).

As used herein, the term "monoclonal antibody (mAb)", shall refer to an antibody composition having a homogenous antibody population, i.e., a homogeneous population consisting of a whole immunoglobulin, or a fragment or derivative thereof. Particularly preferred, such antibody is selected from the group consisting of IgG, IgD, IgE, IgA and/or IgM, or a fragment or derivative thereof.

As used herein, the term "derivative" shall refer to protein constructs being structurally different from, but still having some structural relationship to, the common antibody concept.

Methods for the production and/or selection of chimeric, humanised and/or human mAbs are known in the art. For example, US6331415 by Genentech describes the production of chimeric antibodies, while US6548640 by Medical Research Council describes CDR grafting techniques

and US5859205 by Celltech describes the production of humanised antibodies. In vitro antibody libraries are, among others, disclosed in US6300064 by MorphoSys and US6248516 by MRC/Scripps/Stratagene. Phage Display techniques are for example disclosed in US5223409 by Dyax. Transgenic mammal platforms are for example described in US200302048621 by TaconicArtemis.

The term "new antibody format" encompasses, for example bi- or trispecific antibody constructs, Diabodies, Camelid Antibodies, Domain Antibodies, bivalent homodimers with two chains consisting of scFvs, IgAs (two IgG structures joined by a J chain and a secretory component), shark antibodies, antibodies consisting of new world primate framework plus non-new world primate CDR, dimerised constructs comprising CH3+VL+VH, and antibody conjugates (e.g., antibody or fragments or derivatives linked to a toxin, a cytokine, a radioisotope or a label). This list is however not restrictive.

Further, the term also encompasses immunotoxins, i.e., heterodimeric molecules consisting of an antibody, or a fragment thereof, and a cytotoxic, radioactive or apoptotic factor. Such type of format has for example been developed by Philogen (e.g., anti-EDB human antibody L19, fused to human TNF), or Trastuzumab emtansine (T-DM1), which consists of trastuzumab linked to the cytotoxoic Mertansine (DM1).

The term "fusion peptide" or "fusion protein" proteins relates, for example, to proteins consisting of an immunoglobulin Fc portion plus a target binding moiety (so-called -cept molecules).

In another preferred embodiment of the method or process according to the invention, it is provided that the immunoligand has a reduced degree of fucosylation compared to an immunoligand expressed in the absence of an elevated concentration of manganese or manganese ions.

Preferably, the degree of fucosylation is determined by methods according to the art. Such methods comprise, among others, digestion with Peptide-N-Glycosidase F (PNGase F), to deglycosylate the antibodies (see description at Fig. 1 for more details), and subsequent collection of the isolated glycanes. The collected glycanes are labeled with anthranicilic acid and then analyzed by means of NP HPLC. Full details of the method are disclosed in Anumula (2012), content of which is incorporated herein by reference.

The term "absence of an elevated concentration of manganese or manganese ions." means that during the process or in the preparation of the process, no manganese or manganese ions have willingly been introduced. This does not exclude that traces of manganese naturally occurring in media like water can still be present.

In a preferred embodiment of the method or process according to the invention, it is provided that the immunoligand demonstrates an increased ADCC activity compared to an immunoligand (i) expressed in the absence of an elevated concentration of manganese or manganese ions or (ii) having a higher degree of fucosylation.

The term "ADCC" relates to a mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies. It is one of the mechanisms through which antibodies, as part of the humoral immune response, can act to limit and contain infection. Classical ADCC is mediated by natural killer (NK) cells; macrophages, neutrophils and eosinophils can also mediate ADCC. ADCC is part of the adaptive immune response due to its dependence on a prior antibody response.

Preferably, the ADCC activity is determined by methods according to the art. Such methods comprise, among others, the cytotoxicity assay as shown in Figure 3.

Other suitable assays include include chromium-51 [Cr51] release assay, europium [Eu] release assay, and sulfur-35 [S35] release assay. Usually, a labelled target cell line expressing a certain surface-exposed antigen is incubated with antibody specific for that antigen. After washing, effector cells expressing Fc receptor CD16 are co-incubated with the antibody-labelled target cells. Target cell lysis is subsequently measured by release of intracellular label by a scintillation counter or spectrophotometry. The coupled bioluminescent method aCella TOX is now in widespread use for ADCC and other cytotoxicity assessments. Since this technique measures the release of enzymes naturally present in the target cells, no labeling step is required and no radioactive agents are used.

Preferably, the immunoligand targets one or more cellular surface antigens involved in cell-mediated immune defense.

Preferably, said cellular surface antigens are selected from the group consisting of cyclophilin C, complement factor I, CD6, CD5, bovine WC-1 and M130.

CD6 is an important cell surface protein predominantly expressed by human T cells and a subset of B cells, as well as by some B cell chronic lymphocytic leukemias and neurons (Aruffo et al. 1991, Kantoun et al. 1981, Mayer et al. 1990). CD6 is a member of a large family of proteins characterized by having at least one domain homologous to the scavenger receptor cysteine-rich domain (SRCR) of type I macrophages (Matsumoto et al. 1991 and Resnick et al. 1994). Other members of this family include CD5 (Jones et al., 1986) cyclophilin C (Friedman et al. 1993), complement factor I, which binds activated complement proteins C3b and C4b (Goldberger, et al., J. Biol. Chem. 1987, 262:10065), bovine WC-1 expressed by .tau./.delta. T cells (Wijingaard et al. 1992) and M130 (Law et al. 1993), a macrophage activation marker.

Other preferred surface antigens encompass CD20, EGFR, HER2/neu, and membrane-bound TNF.

In a preferred embodiment of the method or process according to the invention, it is provided that the immunoligand is Itolizumab.

Itolizumab (INN, trade name Alzumab ®) is a 'first in class' humanized IgG1 monoclonal antibody developed by Biocon. It selectively targets CD6, a pan T cell marker involved in co-stimulation, adhesion and maturation of T cells. Itolizumab, by binding to CD6, down regulates T cell activation, causes reduction in synthesis of pro-inflammatory cytokines and possibly plays an important role by reducing T cell infiltration at sites of inflammation. A double blind, placebo controlled, phase III treat –Plaq study of itolizumab successfully met the pre-specified primary end-point of significant improvement in PASI-75 (Psoriasis Area and Severity Index) score after 12 weeks of treatment in patients with moderate to severe psoriasis compared to placebo. Biocon received marketing authorization for the drug from the Drugs Controller General of India (DCGI) in January 2013 and marketing within India commenced in August 2013 (Jayaraman, 2013).

Itolizumab is produced from mouse derived NS0 cell line (called herein "T1h") and also from Chinese Hamster Ovary (CHO) cell line (called herein "Bmab-600"). The Fc portions of Bmab-600 and T1h bind to FcγRIIIa with different affinities as the post translational modifications, especially the afucosylation pattern varies with cell line and culture conditions.

Itolizumab can for example be produced from mouse derived NS0 cell line (called herein "T1h") and also from Chinese Hamster Ovary (CHO) cell line (called herein "Bmab-600"). The Fc portions of Bmab-600 and T1h bind to FcγRIIIa with different affinities as the post translational modifications, especially the afucosylation pattern varies with cell line and culture conditions.

According to another aspect of the invention, a glycoprotein is provided, which glycoprotein is produced with a method or process according to any of the method or process of the invention.

Preferably, said glycoprotein is a recombinant protein. More preferably, said glycoprotein is an immunoligand, preferably an antibody. It is particularly preferred that said glycoprotein has a decreased fucose content in its glycosylation pattern.

Preferably, the glycoprotein, or a subdomain thereof, like an Fc region, has an afucosylation level of around 35%.

In a preferred embodiment, it is provided that the glycoprotein has an increased ADCC. Preferably, said glycoprotein is Itolizumab.

In another preferred embodiment, it is provided that the glycoprotein effects *in vitro*- or *in vivo* reduction of cells being positive for CD25 and CD4, in particular of CD4+ T cells.

The inventors have surprisingly shown that the use of anti-CD6 antibody according to the invention leads to reduced proliferation of cells which are positive for the surface antigens CD25 and CD4 (see Fig. 5B and description), in particular CD4+ T-Cells.

The term "reduction of cells", as used herein, refers to (i) the inhibition of proliferation, (ii) the depletion, (iii) induction of apoptosis or (iv) other mechanisms which lead to a reduction of such cells.

According to another aspect of the invention, the use of a glycoprotein as set forth above for the manufacture of a medicament for the treatment of a human or animal patient is provided. Likewise, the use of a glycoprotein as set forth above for the treatment of a human or animal patient is provided.

In a preferred embodiment of such use, the human or animal patient suffers from or has been diagnosed to be at risk to develop a disease selected from the group consisting of

- Neoplastic diseases, including tumors, lymphomas and leukemias, in particular B-cell chronic Lymphocytic leukemia (B-CLL), particularly T-cell leukemias
- Autoimmune disease, including Rheumatoid arthritis, Psoriasis, Crohn's disease, Lupus erythematosus, and/or Sjogren's disease
- Neurodegenerative diseases, including Multiple sclerosis, and/or Parkinson's disease, Alzheimer's disease, Huntington's disease and/or Amyotrophic lateral sclerosis, and/or
- Infectious diseases

Preferably, such use relates to the treatment or prevention of averse reactions like GVHD (Graft vs. Host disease) in a human or animal that has been transplanted. Such transplantation includes organ transplants as well as bone marrow transplants.

EXPERIMENTS AND FIGURES/EXAMPLES

While the invention has been illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive; the invention is not limited to the disclosed embodiments. Other variations to the disclosed embodiments can be understood and effected by those skilled in the art in practicing the claimed invention, from a study of the drawings, the disclosure, and the appended claims. In the claims, the word "comprising" does not exclude other elements or steps, and the indefinite article "a" or "an" does not exclude a plurality. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage. Any reference signs in the claims should not be construed as limiting the scope.

Figure 1: Results of a deglycosylation experiment carried out with an antibody having an Fc region.

The anti CD antibody Itolizumab (also called T1h), has been incubated with a deglycosylation buffer (50mM Tris, 1mM CaCl2, pH=8.1) in a 1:1 ratio to Itolizumab (5 mg/ml) followed by 24 hours incubation of Peptide-N-Glycosidase F (PNGase) enzyme (10 U for 1 mg antibodies).

After incubating for 24 hrs at 37 °C, an equal volume of T1h buffer (Histidine Trehalose buffer) in sample is added and centrifuged in centricon tubes (50kD cut off filters) at 4 °C, 4000 rpm for 15 minutes. The residual volume is re-suspended again in equal volume with T1h buffer and centrifuged at 4 °C, 4000 rpm for 15 minutes. The de-glycosylated Ab is stored in final storage tube and concentration estimated by Nano drop. The deglycoslation is confirmed by CE-SDS (Capillary Electrophoresis). FACS (Fluorescence activated cell sorter) analysis has then been carried out. Briefly, HUT78 cells (T cells line) are labelled with the anti CD6 antibody T1h, or the deglycosylated T1h antibody produced as described above.

Subsequently coming with a secondary anti Fc antibody, labelled with FITC, a signal is observed. Fig. 1 shows that deglycosylation of the Fc region of Th1 does not compromise its ability to bind to CD6 expressing cell lines. These results have further been confirmed by Plasmon resonance experiments.

Figure 2: Result of a deglycosylation experiment carried out with an antibody having an Fc region.

In particular, the anti CD antibody Itolizumab was deglycosylated as discussed *supra*. It's abilty to inhibit of proliferation of activated T cells was then compared with that of unmodified T1h in a suitable proliferation assay. Nimotuzumab, which is an antibody that has the same IgG backbone as that of Itolizumab but binds to EGFR, was used as negative control.

Briefly, the antibody was coated on sterile 96 well plates in a concentration range 0-1μg/ml overnight with bicarbonate buffer at pH9.5. After washes purified lymphocytes from normal healthy volunteers were added to the plates. Itolizumab from 80-1μg/ml was added and the culture was incubated for 4 days. Alamar blue was added to measure proliferation. Fold difference is calculated relative to unstimulated cells control. Isotype Nimotuzumab antibody was used as control..Plate bound anti CD3 (the anti CD3 used is OKT3 clone manufactured at center for molecular immunology, Cuba) stimulates the proliferation of naïve T cells (Peripheral Blood Mononuclear Cells (PBMC) from a human donor, purified over a density gradient of Ficoll) from normal healthy volunteers.

Nimotuzumab (80μg/ml) does not show any inhibition of the T-Cell proliferation, resulting in about 2.75 fold increase in cells relative to unstimulated cells, while native T1h shows inhibition of the T-Cell proliferation (35 – 20 % inhibition in the 80 μg/ml - 1.25μg/ml range). In contrast thereto, the impact of deglycosylated T1h is similar to that of Nimotuzumab. This means that upon deglycosylating, the antibody loses its ability to inhibit the proliferation of T cells.

Figure 3: Results of a cytotoxicity assay comparing native antibody and deglycosylated antibody.

Frozen PBMCs were thawed in RPMI 1640 Media with 10% FBS in presence of IL-2 (Conc.2.5ng/mL) and incubated overnight in a 37°C, 5% CO_2 incubator. On the next day cells

were resuspended in media without IL-2 and incubated for 4-5 hrs. In a 96 well plate 12,000 Hut-78 cells/50µLwere added to each well. 50 µL 3X concentrated drug (either native T1h, deglycosylated T1h or anti CD3 at 10microgram/ml) as per template were added and incubated for 2 hours at 37°C, 5% CO_2 incubator. PBMCs were resuspended in assay media and 240,000 PBMCs/50µL/well were added, to obtain a target to effector ratio of 1:20. The plates were incubated at 37°C, 5% CO_2 incubator for 22 hours. 50µL of Cyto Tox-Glo was added to the plates and incubated for 30 minutes at room temperature. The plates were read using Spectramax for luminescence to determine the cytotoxicity.

While native T1h shows mild but statistically consistent Antibody Dependent Cellular Cytotoxicity (ADCC) activity relative to anti CD3, which is a partially depleting antibody targeting T cells, this ADCC activity is significantly reduced on deglycosylation of the molecule, indicating the effector function of T1h. Use of Fab2 fragment of Itolizumab can also reduce the ADCC activity comparable to the deglycosylated molecule.

Figure 4: Results of a Mixed Lymphocyte Reaction (MLR) experiment comparing native antibody and deglycosylated antibody.

Preparation of PBMCs: 30 ml of blood was collected from a healthy donor. PBMCs were isolated by standard FICOLL density gradient centrifugation Monocyte Depletion & Setting up

Dendritic Cell (DC) Derivation Assay: These cells were incubated in a CO_2 incubator for two hours. Monocytes were allowed to adhere onto the plastic surface. The non-adhered cells (PBLs) were subsequently removed from the flasks. All the flasks were washed with 1XPBS once. 20 ml of DC media (made 50 ml stock, 10 µl of Granulocyte macrophage colony-stimulating factor (GMCSF) and 5 µl of IL-4 in 50 ml of assay media) was added to each flask. The flasks were kept in CO_2 incubator for 6 days.

LPS Treatment to on-growing Dendritic Cells: At day 6, DC media with LPS (Lipopolysaccharides) was added to each flask (final concentration of LPS in the flask is 4 ug/ml) and kept back in CO2 incubator for 40 -- 48 hrs.

Preparation of DCs: After LPS treatment the cell suspension (DC) was collected from the two flasks. Each flask was washed with 1XPBS once. The cell suspension was spun down at 1500 rpm for 5 minutes and reconstituted in 3 ml media. LPS treated DCs were counted and reconstituted in media as per assay requirement.

Preparation of PBLs: Following the same protocol as mentioned before, Ficoll separation was performed after collecting blood from another healthy individual. After monocyte depletion the non adhered Peripheral blood lymphocytes (PBLs) were collected and spun down at 1500 rpm for 5 minutes and reconstituted in 5 ml media. PBLs were counted and reconstituted to 1.0×10^6 cells/ml.

SEB treatment to Dendritic Cells (DC): Staphylococcal enterotoxin B (SEB) stock concentration is 1mg/ml. From the stock 3 µl of SEB is dissolved in 3 ml of media to get 1 mg/ml working solution of SEB. As per the standardized protocol 0.06×10^6 DCs are treated with 0.6 ug of SEB. A stock 0.1x106 cells/ml (LPS treated matured DCs) is made. From this, 600 µl of cell suspension is dissolved in 2.4 ml of assay media (total volume of cell suspension is 3 ml that contains 0.02×10^6 cells/ml). This is spun down at 1500 rpm for 5 min and 600 ml of SEB (1ug/ml) is added to the pellet. This is incubated inside CO2 incubator at 37°C for 20 minutes. Excess media (2 ml) is added to the tube after incubation and washed at 1500 rpm for 5 min. Supernatant is discarded and the cells are washed again with 3 ml of media. Finally the pellet is dissolved in 3 ml of assay media.

Mytomycin C treatment to PBLs: 25 µg/ml Mytomycin solution is made from the Mytomycin stock of 1mg/ml. $0.5X10^6$ PBLs are treated with 500 µl of 25µg/ml Mytomycin for 30 min inside CO_2 incubator at 37°C. Excess media (2 ml) is added to it after the incubation and the cells are washed at 1500 rpm for 5 media. Supernatant is discarded and the cells are washed again with 3 ml of media.

MLR Assay - Inhibition of Proliferation: MLR assay is performed at DC:PBL = 1:50 ratio. Negative control used is Nimotuzumab. Native T1h was tested against a Fab2 version thereof which lacks the fully functional Fc region. After 6 days the plate is read with alamar blue using Bio-Tek Synergy HT Gen5 plate reader.

While the intact antibody can inhibit the proliferation of T cells induced in this reaction, negative control Nimotuzumab with different specificity cannot. T1h without the Fc region cannot inhibit the T cell proliferation either, suggesting that the glycosylated Fc region along with Fab is critical for the inhibitory capacity of T1h in this assay. A similar effect has also been observed with the use of a deglycosylated T1h thereby confirming the need of the glycosylation for the effector function of T1h

Figure 5a: Results of another Mixed Lymphocyte Reaction (MLR) experiment comparing different immunomodulators.

The protocol is identical to Figure 4. It is a mixed lymphocyte reaction. In addition to native T1h at four concentrations other immunosuppressant and immunomodulators were used, namely

pimecrolimus (Pim), Abatacept (Aba) and Daclizumab (Dac) are included as positive controls for the assay. Nimotuzumab (hR3) is used as a negative control.

It turned out that T1h is able to reduce the proliferation of T cells induced in a mixed lymphocyte reaction as compared to an isotype antibody, Nimotuzumab binding to Human EGFR. The fold reduction induced by T1h is comparable to that induced by Abatacept (CTLA4-IgG1Fc), Daclizumab (Anti CD25) and Pimecrolimus (small molecule, IL2 inhibitor).

Figure 5b: Analysis of the experiments shown in Fig. 5a.

The analysis relates to cells from the culture after 144 hours (6 days) in the mixed lymphocyte reaction. B--, B++, B+- and B-+ are are the quadrants. Here the cells in culture in an MLR are evaluated after 6 days. Although the inhibitory capacity of T1h compares well with other antibodies, the path is different for T1h as here unlike with the other molecules there is a significant decrease in CD4/ CD25 activated T cell population. T1h shows reduction in CD25+, CD4+ as well as CD4+ T cells. This indicates selective depletion of a subset of T cells. Hence, although as shown in Figure 5A, the inhibition in the MLR by T1h is comparable to that of Daclizumab, Abatacept and Pimecrolimus, only T1h shows a decrease in CD25+, CD4+ as well as CD4+ T cells.

Figure 6: Results of a cytotoxicity assay comparing native antibody and deglycosylated antibody.

The same assay in Figure 3 was used to evaluate the antibodies with different afucosylated content, compared to the positive control, anti-CD3. The data shown is a compilation from n=4 independent experiments.

Afucosylation took place as described elsewhere herein (see, e.g., description of Fig. 11). Increased afucosylation of the Fc region of Itolizumab shows a linear increase in the ADCC activity exhibited by the antibody with respect to the positive control antibody (anti-human CD3). This demonstrates the ability of Itolizumab to be more cytotoxic by merely increasing the afucosylated Fc Glycan content. For example, to enhance the ADCC from 20 % relative to that of anti CD3 to greater than 40 %, the afucosylated content in the antibody should be greater than 10%.

Such increase may be caused by better binding to FcγRIII as shown in the biacore data discussed *infra* (wherein Bmab 600 binds with better affinity as compared to T1h). Hence increasing the afucosylated species in the antibody can cause better binding to FcγRIII and this translates into a functional activity of better ADCC.

Figure 7: Results of a CDC assay comparing T1h and Rituximab

The Human T cell lymphoma cell line Hut 78 (ATCC® TIB-161™), was used to assess the CDC activity of T1h. $1x10^4$ cells were incubated with the respective drug dilutions at 10µg/mL, 1µg/mL and 0.01µg/mL for 20 minutes in a 37°C, 5% CO_2 incubator. Pooled normal human serum was added at a final concentration of 1:10 and cells were incubated for 2 hours at 37°C. alamarBlue® (Invitrogen) was added and cells were incubated for 20 – 22 hours at 37°C. The uptake of the dye by cells, followed by its reduction is read as fluorescence at 530/590 nm.

Rituximab, an anti CD20 targeting CD20 receptors on a B cell line (Daudi) and causing *complement*-dependent cytotoxicity (CDC) was used a s a positive control in the assay to show that the serum components resulting in CDC was intact.

T1h does not exhibit CDC activity. Increase in the afucosylated species of Itolizumab does not increase the CDC activity of the molecule, concluding that only ADCC effector functions are enhanced with increase in afucosylation.

Batch	G0-GN	G0f-GN	G0	G0f	Man5	G1f-GN,G1	G1f	Man6,(G1f-GN)S1	G1FS1,Triantennary complex with 1 G, hybridS1	G2f	G2fS1, with small hybrid	G2fS2	Others hybrid species	Total non fucosylated species
T1h Range	0.1-0.5	0.8-2.2	0.1-0.3	23.5-36.7	1.2-3.7	1.2-3.4	37.9-43.8	0.6-1.4	1.6-2.6	8.2-12.2	6.5-13.6	1.5-3.8	0.5-1.3	3.95
1185/12/03/25D	0.7	2.2	5.1	45.1	3.0	3.4	31.3	1.3	1.0	4.2	1.9	0.2	0.7	10.1
1185/12/03/27D	0.5	1.5	4.3	42.7	2.6	3.6	34.4	1.3	1.0	5.0	2.1	0.1	0.8	8.7
1185/12/03/34D	0.5	1.3	4	45.1	6.8	2.9	29.2	1.8	0.9	4.2	1.9	0.7	0.9	13.1
1185/12/03/31D	0.6	1.5	4.5	42.4	7.3	3.7	29.1	2.1	1.0	4.0	2.3	0.3	1.1	14.5
1185/11/01/35D	0.7	1.4	6.5	46.7	4.8	3.5	28.2	1.4	0.8	3.4	1.3	0.6	0.7	13.4

Table 1: Glycan profile of differentially afucosylated T1h samples used in assays shown in Figs. 6 and 7.

The analysis of Glycosylation patterns took place with standard methods. In brief, the antibodies were digested with Peptide-N-Glycosidase F (PNGase F), to deglycosylate the antibodies (see description at Fig. 1 for more details), and the isolated glycanes were collected. The collected glycanes were labeled with anthranicilic acid and then analyzed by means of NP HPLC. Full details of the method are disclosed in Anumula (2012), content of which is incorporated herein by reference.

In this table, the following abbreviations are used: G0 = no Galactose, G1 = 1 terminal Galactose residue, G2 = 2 terminal Galactose residues, GN = N-Acetyl Glucosamine or GlcNac, F = Fucose, Man5 = 5 mannose residues, Man6 = 6 mannose residues and S = Sialic acid.

An explanation of the Glycosylation patterns determined in the course of the experiments shown herein, and the nomenclature used, is provided in Fig. 15.

Figures 8 - 10: Binding curves of T1h to FcγRIIIa detect with Plasmon resonance

BIAcore is an analytical device which detects differences in surface plasmon resonance-based changes in the refractive index near a sensor surface. This method of determining affinity constants of an antibody for Fc receptors ligands has been used widely. In order to detect an interaction one molecule (the ligand) is immobilized onto the sensor surface. Its binding partner (the analyte) is injected in aqueous solution (sample buffer) through the flow cell, also under continuous flow. As the analyte binds to the ligand, the accumulation of protein on the surface results in an increase in the refractive index, which is plotted against time to yield a sensorgram. Association (K_a), dissociation-rate constants (K_d) and equilibrium dissociation constants (K_D) are determined from the analysis of sensorgrams.

FcγRIIIa is considered as an intermediate affinity receptor. It can variably bind monomeric IgG and appears to have a high affinity for IgG than the lower affinity Fc gamma receptors. They are expressed on the NK cells and monocytes of the blood cells.

The Fc portions of Bmab-600 and T1h bind to FcγRIIIa with different affinities as the post translational modifications, especially the afucosylation pattern varies with cell line and culture conditions. We evaluated these two products binding affinities towards FcγRIIIa in Biacore instrument. The binding affinity results of Bmab-600 show higher affinity in binding to FcγRIIIa receptors in comparison to T1h. The following samples were analyzed on the surface immobilized with FcγRIIIa receptor:

1. T1h antibody
2. Bmab-600 antibody
3. Deglycosylated T1h antibody

Each sample was analyzed two times and the average K_D (μM) values are reported and compared against each other. Figure 8 shows the binding curves of T1h antibody to FcγRIIIa, Figure 9 shows the binding curves of Bmab-600 antibody to FcγRIIIa, and Figure 10 shows the binding curves of deglycosylated T1h antibody to FcγRIIIa.

The method was sensitive and was able to pick-up the differences between afucosylation differences that were existing inherently in the differentially afucosylated samples of Bmab-600 and T1h. The data also shows that as the afucosylation levels increases the FcγRIIIa binding affinity values decreases (meaning higher affinity) proportionally. The method specificity was also demonstrated by analyzing the deglycosylated sample of T1h where no binding interactions was observed.

Samples	ka (1/Ms)	kd (1/s)	K_D (M)	K_D (μM)	Average K_D (μM)
	1.52E+04	5.65E-03	3.72E-07	0.372	0.440

T1h (NS0)	1.16E+04	5.88E-03	5.08E-07	0.508	

Table 2: Kinetic values of T1h antibody to FcγRIIIa (see Fig. 8)

Samples	**ka (1/Ms)**	**kd (1/s)**	**K_D (M)**	**K_D (μM)**	**Average K_D (μM)**
Bmab-600 (CHO)	2.46E+04	4.76E-03	1.93E-07	0.193	0.200
	2.78E+04	5.73E-03	2.07E-07	0.207	

Table 3: Rate constant values of Bmab600 antibody to FcγRIIIa (see Fig. 9)

Samples	**ka (1/Ms)**	**kd (1/s)**	**K_D (M)**	**K_D (μM)**	**Average K_D (μM)**
T1h (NS0)	Negative interaction				

Table 4: Rate constant values of deglycosylated T1h antibody to FcγRIIIa (see Fig. 10)

Samples	**% afucosylation**	**ka (1/Ms)**	**kd (1/s)**	**K_D (M)**	**K_D (μM)**
T1h	2.5	1.18E+04	5.54E-03	4.686E-07	0.468
Bmab-600	5.1	1.28E+04	4.20E-03	3.28E-07	0.328
Bmab-600	9.6	1.96E+04	6.00E-03	3.06E-07	0.306

Bmab-600	35.6	2.73E+04	5.34E-03	1.95E-07	0.195

Table 5: Rate constant values of differentially afucosylated samples of Bmab-600 and T1h antibody to FcγRIIIa (see Fig. 10)

Figure 11: Afucosylation caused by addition of manganese (Mn)

Addition of Mn at concentrations higher than the media concentration (0.005μM) was tested for a CHO-S cell line producing T1h monoclonal antibody. The trials were started with initial cell count of 0.8-0.9 million cells/ml. Regular feeding of glucose and amino acids was carried out during the process to meet the nutritional requirement of cells. Periodic samples were taken to check the cell growth, viability and IgG titre profiles. The broths were harvested at the end of the culture and analyzed for glycosylation profiles as described elsewhere herein.

The trials were done in 2 sets. The first set was carried out in shake flasks and the second set was performed in 50L bioreactors. Manganese was added in culture medium and through feed at specified intervals during the run.

Figure 11 shows an increase in afucosylation level with addition of Manganese in the culture medium and through feed. The afucosylation profiles correspond to day 10 sample in case of 0.1mM, 0.2mM and 0.25mM; and day 11 in case of 0.075mM and 0.23mM. The results are summarized in the following tables:

Mn concentration tested	G0-GN	G0f-GN	G0	G0f	Man5	G1f-GN,G1	G1f	Man6, (G1f-GN)S1	G1FS1,Tri antennary complex with 1 G, hybridS1	G2f	G2fS1, with small hybrid	G2fS2	Other hybrid species	Afucosylation
0.1mM	0.5	1.1	4.9	56.7	3.1	1.9	25.2	1.1	0.8	2.5	1.2	0.5	0.5	9.6
0.2mM	0.6	0.9	6.3	55.1	4.2	2.3	24.1	1.2	0.8	2.4	1.1	0.5	0.7	12.3
0.25mM	1.2	1	10.3	51.2	6.8	3.1	20.3	1.1	0.7	1.9	1	0.4	1	19.4

Table 6: Glycan profile of a 10-day shake flask trial with 0.1mM, 0.2mM and 0.25mM Manganese (Mn) concentrations

Mn concentration tested		G0f-GN	G0	G0f	Man5	G1f-GN,G1	G1f	Man6, (G1f-GN)S1	G1FS1,Tri antennary complex with 1 G,	G2f	G2fS1, with small hybrid	G2fS2	Others hybrid species	Afucosylation
0.075mM	0.2	1	2	40.6	1.9	2.3	38.9	1.8	1.1	6.2	2.3	0.9	0.8	5.9
0.23mM	0.6	1.5	4.5	42.4	7.3	3.7	29.1	2.1	1	4	2.3	0.3	1.1	14.5

Table 7: Glycan profile of 50L batches run for 11 days with 0.075mM and 0.23mM Mn concentrations

Based on the above experiments, an increase in % afucosylation was observed with increase in total manganese concentration. The cell growth, viability and IgG titre profiles were not affected by Mn addition.

Figure 12: Increase in G0, Man5 and afucosylation levels by addition of manganese (Mn)

The Effect of manganese in the range of 0.0025 μM to 0.5 mM was tested by varying the concentration in culture medium. No manganese addition was done through feeds. The trial was carried out in shake flasks and samples were analyzed for glycosylation profiles on day 8. A a gradual increase in G0, Man5 and afucosylation levels with an increase in manganese concentration could be observed.

Figure 13: Copper concentration does not have an effect on fucosylation.

To evaluate the effect of other divalent cations, Cu was selected for the study since Cu was also a co-factor in the glycosylation pathway (for enzyme Sialyltransferase). Different concentrations of copper in culture medium in the range of 0.01μM to 200μM were tested in shake flasks. No increase/effect in any of the values (G0, Man5 and afucosylation) was observed, as shown in Figure 3. This establishes that copper ions does not affect afucosylation levels in proteins.

Figure 14: Schematic representation of an immunoglobulin G

Fig. 14 shows a schematic representation of an immunoglobulin G (IgG). An IgG is composed of two identical light chains (each composed of two domains, V_L and V_H) and two identical heavy chains (each composed of four domains, V_H, C_H1, C_H2 and C_H3). Antigen binding surface is formed by the variable domains of heavy and light chains and the effector function, such as complement activation and binding of cytotoxic cells is mediated by the Vc region of the antibody.

Figure 15: Nomenclature of N-glycan structures

Fig. 15 shows an overview of different N-glycans. Generally, the term "N-glycosylation" refers to glycosylation of the amino acid residue asparagine (N). Here, an oligosaccharide chain is attached by oligosaccharyltransferase to those asparagine residues which occur in the tripeptide sequences Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except Pro.

The experiments shown herein clearly demonstrate that

a) the fucose content of glycoproteins can be manipulated by varying the total concentration of manganese or manganese ions in media and feeds in the protein expression process

b) increasing total concentration of manganese or manganese ions leads to an increased afucosylation, or to a decreased fucose content in the glycosylation pattern of glycoproteins.

c) in immunoligands like antibodies having an Fc region, protein expression in the presence of an elevated concentration of manganese or manganese ions leads to (i) a higher degree of afucosylation and (ii) an increased ADCC

d) in these immunoligands, increasing the degree of afucosylation does not lead to an increased CDC

e) deglycosylation of immunoligands like antibodies having an Fc region, by contrast, does not lead to an increased ADCC

f) other than afucosylation, deglycosylation of immunoligands like antibodies having an Fc region can lead to loss of functional activity of such immunoligands, in particular if such functional activity is related with activity like effector function and/or ADCC.

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CLAIMS

What is claimed is:

1. A method or process for modifying fucosylation in a eukaryote and/or a eukaryotic protein expression system is provided, in which method or process the total concentration of manganese or manganese ions in the medium is controlled.

2. The method or process according to claim 1, which method or process is a method or process to decrease fucosylation, and in which method or process the protein expression and/or post-translational modification is carried out in the presence of an elevated total concentration of manganese or manganese ions.

3. The method or process according to any of the aforementioned claims, in which method the elevated concentration of manganese or manganese ions is in the range of $\geq$ 0.05 mM - $\leq$ 10 mM.

4. The method or process according to any of the aforementioned claims, in which method the protein expression and/or post-translational modification is carried out in a protein expression system selected from the group consisting of

- Insect cells
- Fungal cells
- Yeast cells

- Protozoan cells, and/or
- Mammalian cells

5. The method or process according to any of the aforementioned claims, in which method the protein is a glycoprotein.

6. The method or process according to any of the aforementioned claims, in which method the protein is a recombinant protein.

7. The method or process according to any of the aforementioned claims, in which method the protein is an immunoligand.

8. The method or process according to any of the aforementioned claims, in which method the immunologand is at least one selected from the group consisting of

- a monoclonal antibody (murine, chimeric, humanized, human), or derivative thereof
- a new antibody format
- a fusion peptide consisting of an immunoglobulin Fc region fused to a target binding moiety, e.g, a receptor fragment

9. The method or process according to any of the aforementioned claims, in which method the immunoligand has a reduced degree of fucosylation compared to an immunoligand expressed in the absence of an elevated concentration of manganese or manganese ions.

10. The method or process according to any of the aforementioned claims, in which method the immunoligand demonstrates an increased ADCC activity compared to an immunoligand (i) expressed in the absence of an elevated concentration of manganese or manganese ions or (ii) having a higher degree of fucosylation.

11. The method or process according to any of the aforementioned claims, in which method the immunoligand targets one or more cellular surface antigens involved in cell-mediated immune defense.

12. The method or process according to any of the aforementioned claims, in which method the immunoligand is Itolizumab.

13. A glycoprotein produced with a method or process according to any of the above claims.

14. The glycoprotein according to claim 13, which glycoprotein is a recombinant protein.

15. The glycoprotein according to any of claims 13-14, which glycoprotein is an immunoligand, preferably an antibody.

16. The glycoprotein according to any of claims 13-15, which glycoprotein, or a subdomain thereof, has a decreased fucose content in its glycosylation pattern.

17. The glycoprotein according to any of claims 13-16, which glycoprotein, or subdomain thereof, has an afucosylation level of around 35%.

18. The glycoprotein according to any of claims 13-17, which glycoprotein has an increased ADCC.

19. The glycoprotein according to any of claims 13-18, which glycoprotein is Itolizumab.

20. The glycoprotein according to any of claims 13-19, which glycoprotein effects an *in vitro*- or *in vivo* reduction of cells being positive for CD25 and CD4, in particular of CD4+ T cells.

21. Use of a glycoprotein according to any of claims 13-20 for the manufacture of a medicament for the treatment of a human or animal patient

22. Use of a glycoprotein according to any of claims 13-20, for the treatment of a human or animal patient.

23. Use according to any of claims 21 - 22, wherein the human or animal patient suffers from or has been diagnosed to be at risk to develop a disease selected from the group consisting of

- Neoplastic diseases, including tumors, lymphomas and leukemias, in particular B-cell chronic Lymphocytic leukemia (B-CLL) and T-cell leukemia
- Autoimmune disease, including Rheumatoid arthritis, Psoriasis, Crohn's disease, Lupus erythematosus, Sjogren's disease
- Neurodegenerative diseases, including Multiple sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease and/or Amyotrophic lateral sclerosis.
- Infectious diseases

24. Use according to any of claims 21 – 23 in the treatment or prevention of averse reactions like GVHD (Graft vs. Host disease) in a human or animal that has been transplanted.

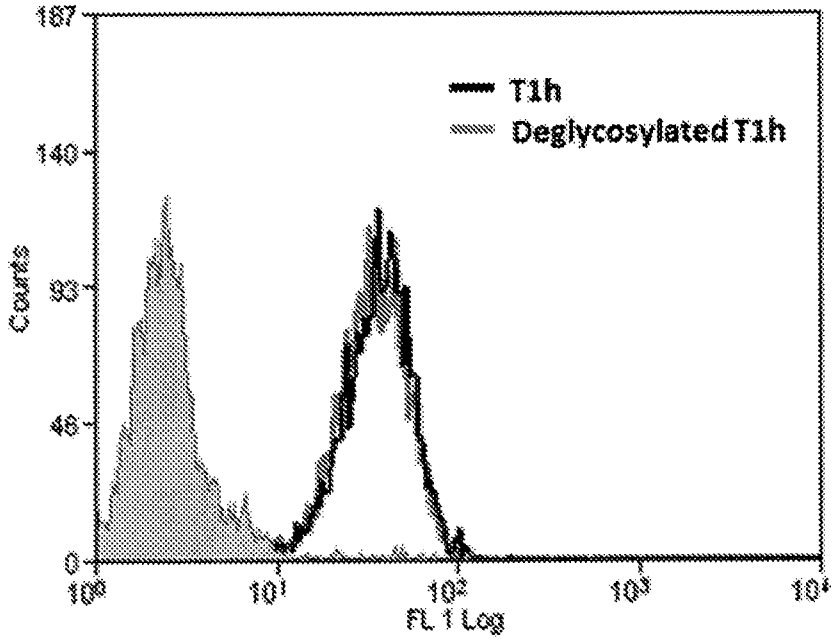

187
140
93
46
0
Counts
T1h
Deglycosylated T1h
10^0
10^1
10^2
10^3
10^4
FL 1 Log

Fig. 1

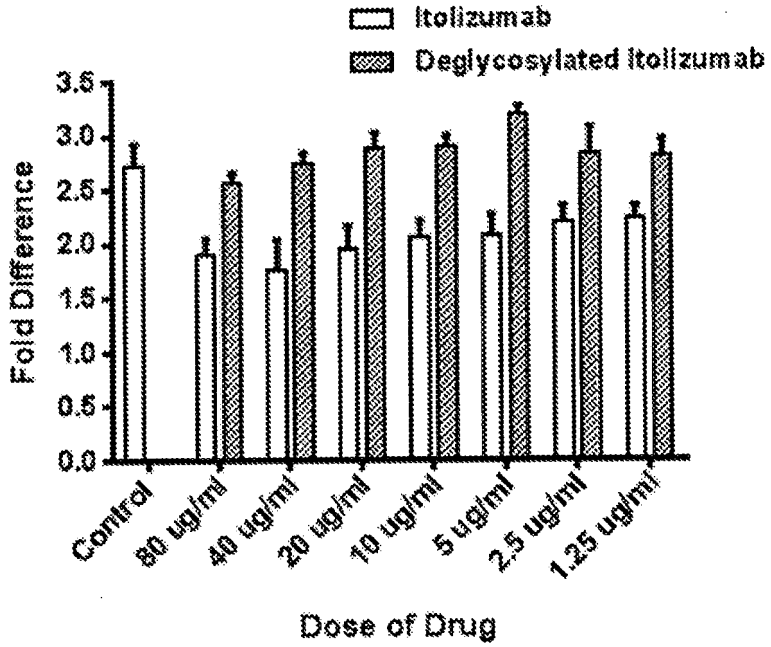

Itolizumab
Deglycosylated Itolizumab
Fold Difference
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
80 ug/ml
40 ug/ml
20 ug/ml
10 ug/ml
5 ug/ml
2.5 ug/ml
1.25 ug/ml
Dose of Drug

Fig. 2

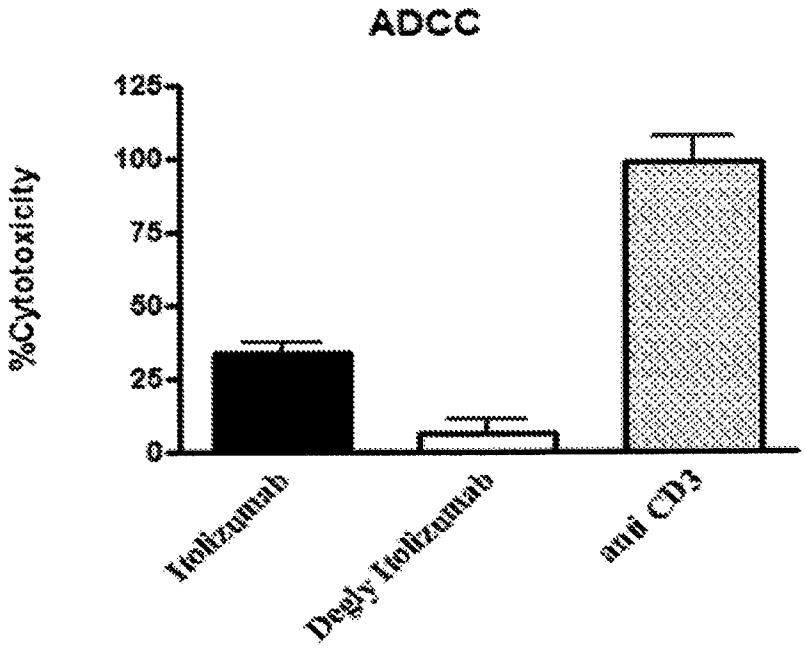

ADCC
%Cytotoxicity
125
100
75
50
25
0
Ioilizumab
Deglyc Ioilizumab
anti CD3

Fig. 3

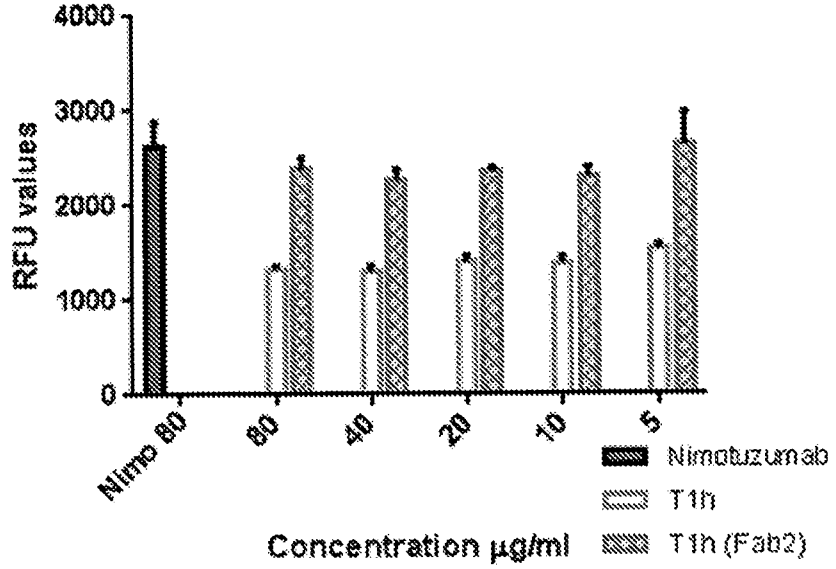

4000
3000
2000
1000
0
RFU values
Nimo 80
80
40
20
10
5
Concentration µg/ml
Nimotuzumab
T1h
T1h (Fab2)

Fig. 4

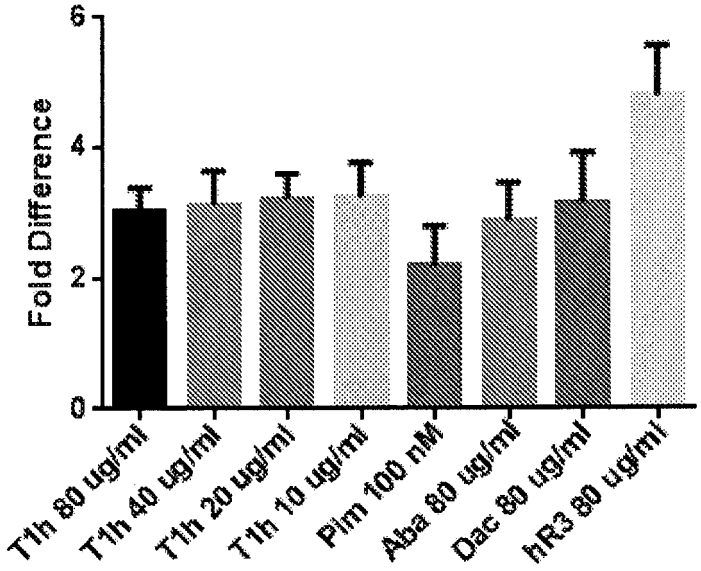

Fold Difference
6
4
2
0
T1h 80 ug/ml
T1h 40 ug/ml
T1h 20 ug/ml
T1h 10 ug/ml
Pim 100 nM
Aba 80 ug/ml
Dac 80 ug/ml
hR3 80 ug/ml

Fig. 5a

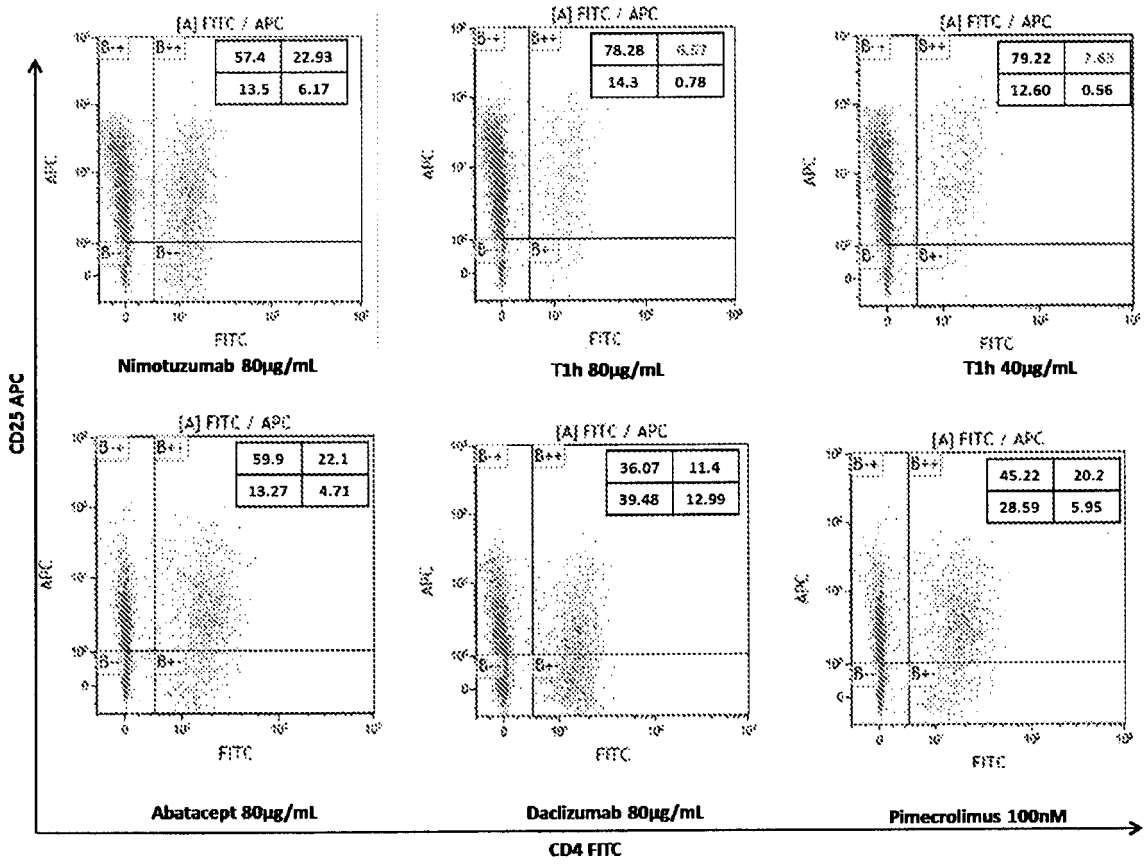

CD25 APC
CD4 FITC
[A] FITC / APC
57.4
22.93
13.5
6.17
Nimotuzumab 80µg/mL
78.28
14.3
0.78
T1h 80µg/mL
79.22
12.60
0.56
T1h 40µg/mL
59.9
22.1
13.27
4.71
Abatacept 80µg/mL
36.07
11.4
39.48
12.99
Daclizumab 80µg/mL
45.22
20.2
28.59
5.95
Pimecrolimus 100nM

Fig. 5b

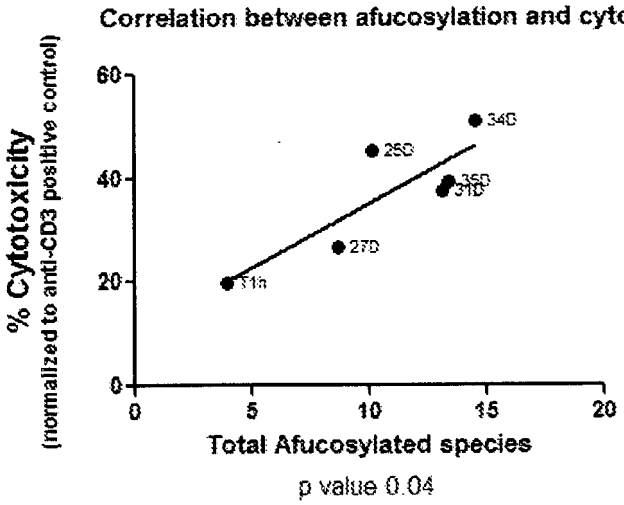

Correlation between afucosylation and cytotoxicity
% Cytotoxicity
(normalized to anti-CD3 positive control)
60
40
20
0
0
5
10
15
20
Total Afucosylated species
p value 0.04
34D
26D
35D
31D
27D
11a

Fig. 6

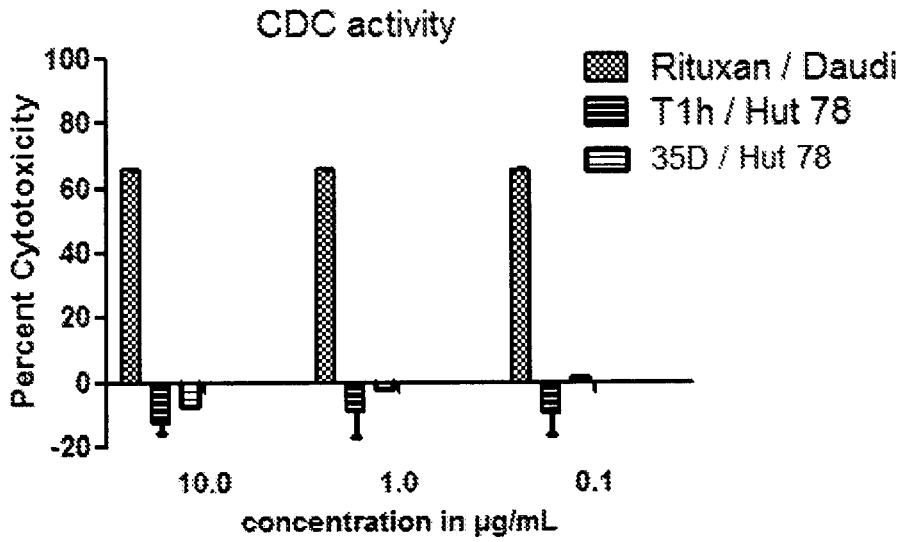

CDC activity
Rituxan / Daudi
T1h / Hut 78
35D / Hut 78
Percent Cytotoxicity
100
80
60
40
20
0
-20
10.0
1.0
0.1
concentration in µg/mL

Fig. 7

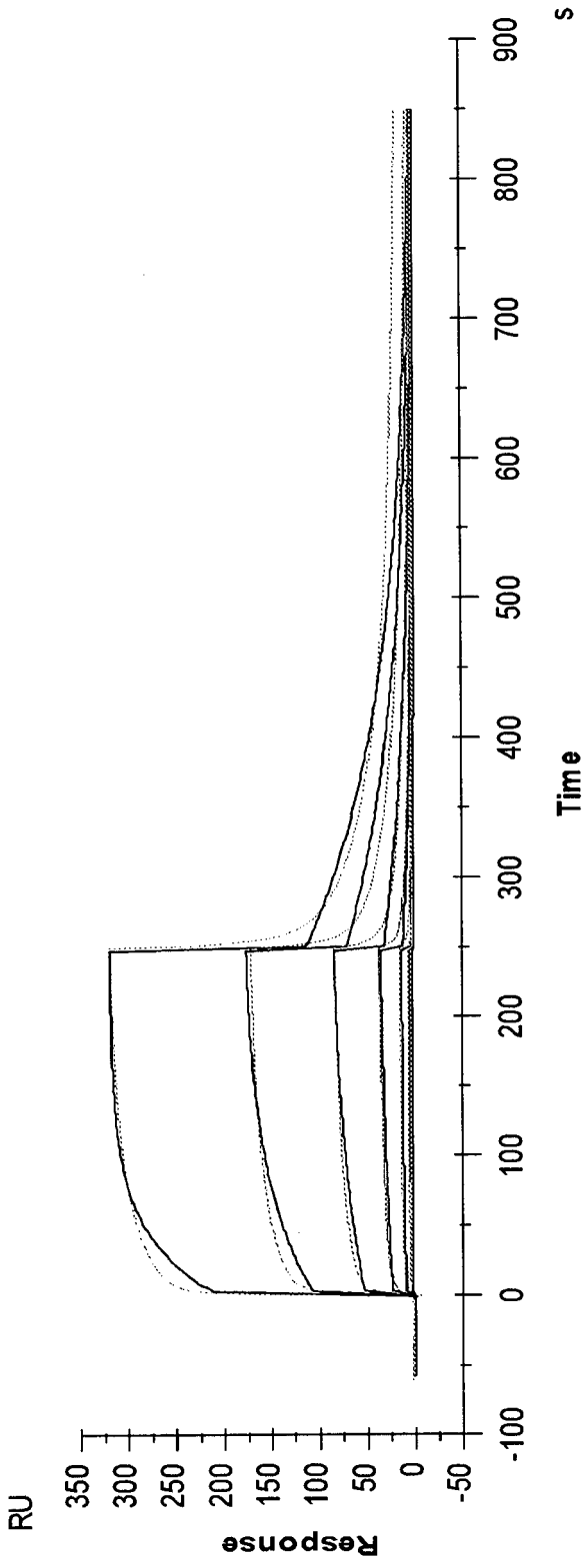

RU
350
300
250
200
150
100
50
0
-50
Response
-100
0
100
200
300
400
500
600
700
800
900
s
Time

Fig. 8

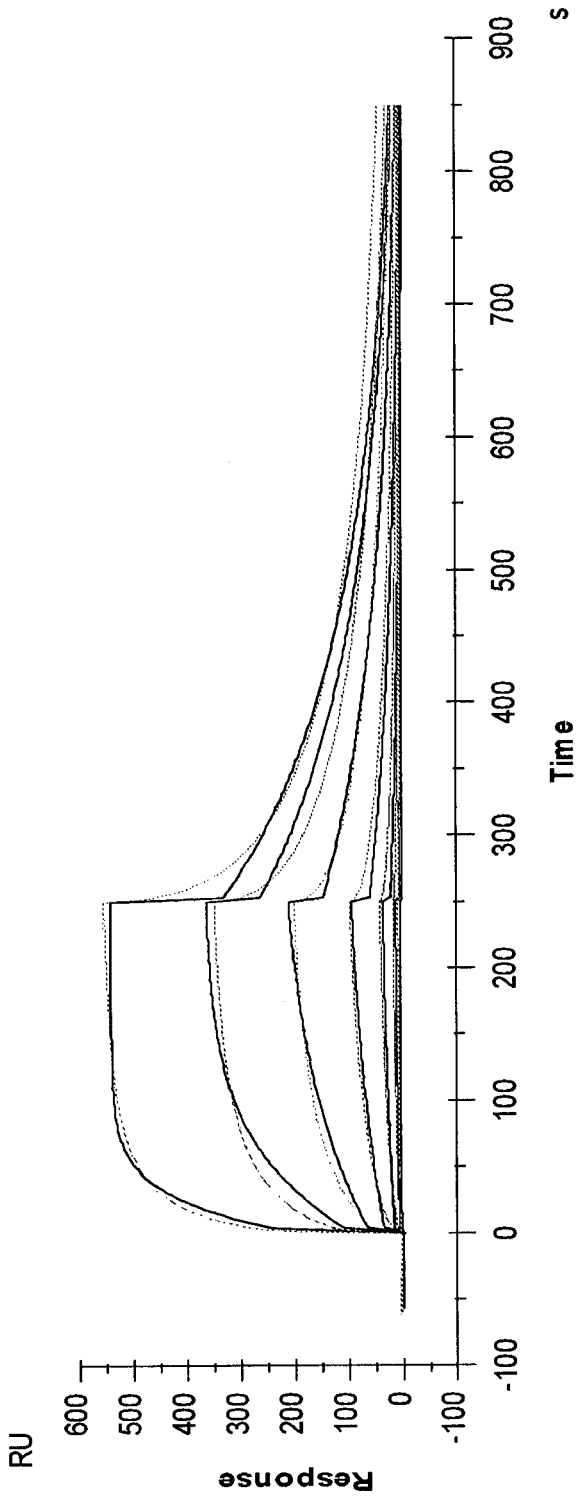

RU
600
500
400
300
200
100
0
-100
Response
-100
0
100
200
300
400
500
600
700
800
900
s
Time

Fig. 9

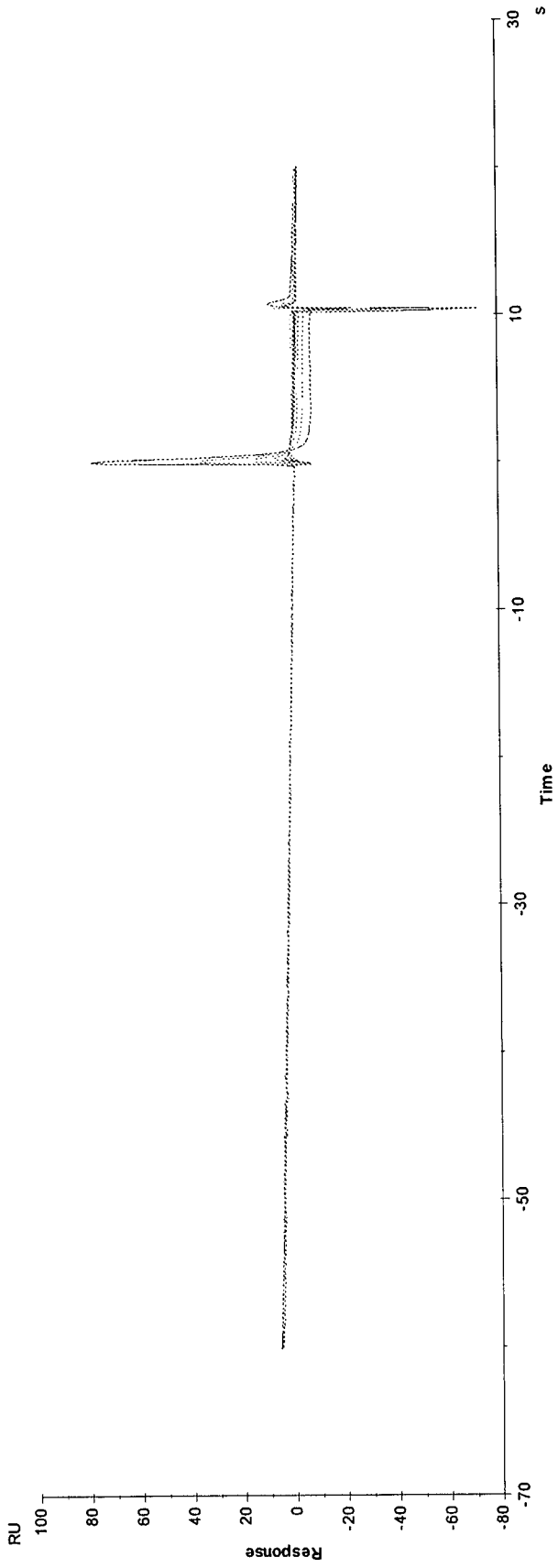


Fig. 10

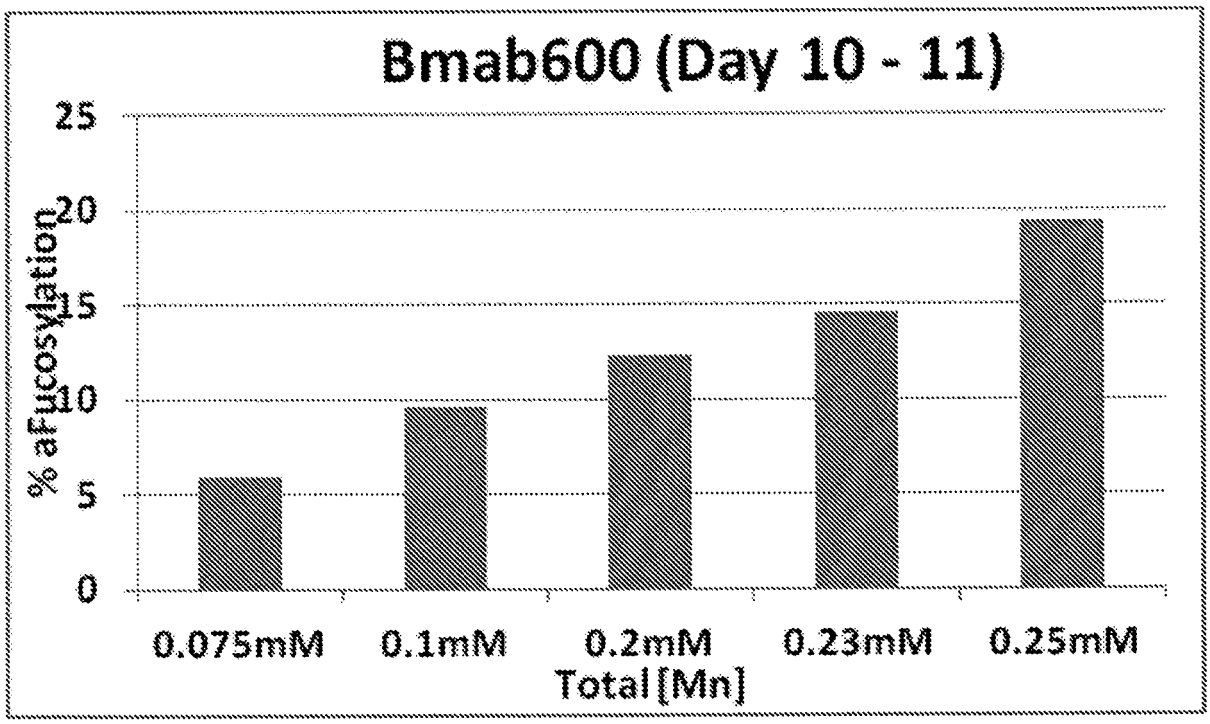

Bmab600 (Day 10 - 11)
% afucosylation
25
20
15
10
5
0
0.075mM
0.1mM
0.2mM
0.23mM
0.25mM
Total [Mn]

Fig. 11

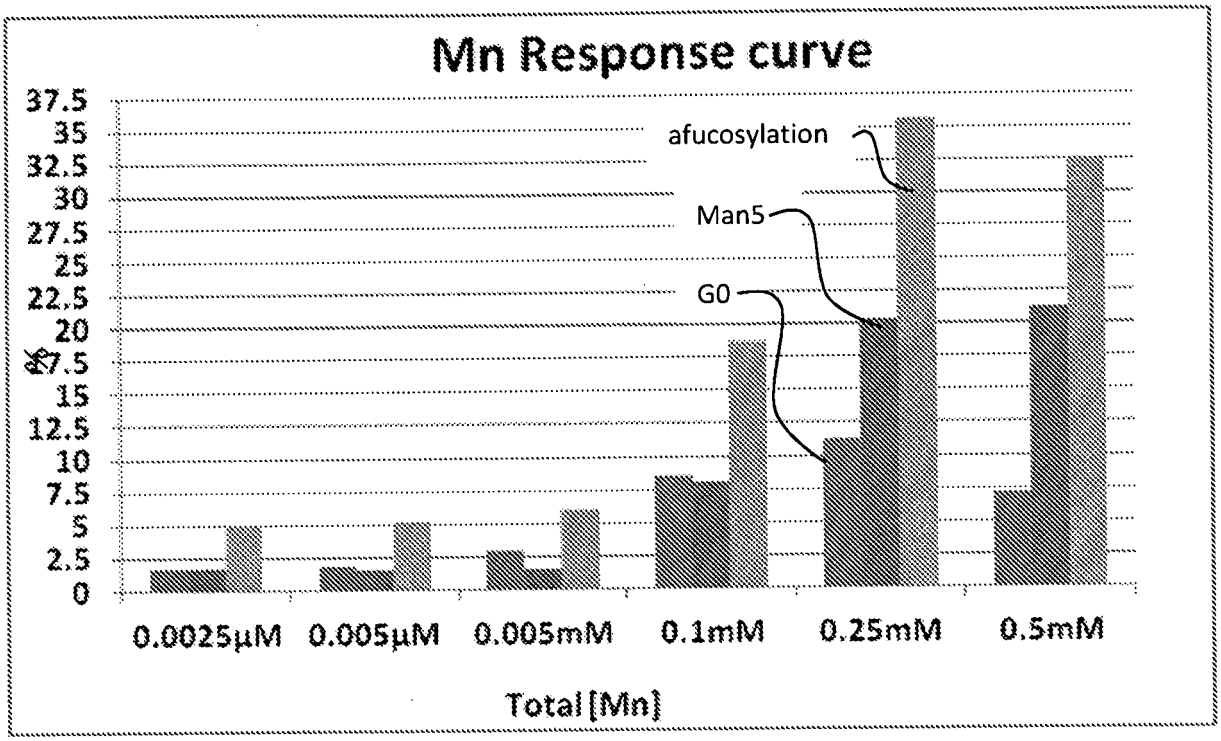

Mn Response curve
37.5
35
32.5
30
27.5
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
afucosylation
Man5
G0
0.0025µM
0.005µM
0.005mM
0.1mM
0.25mM
0.5mM
Total [Mn]

Fig. 12

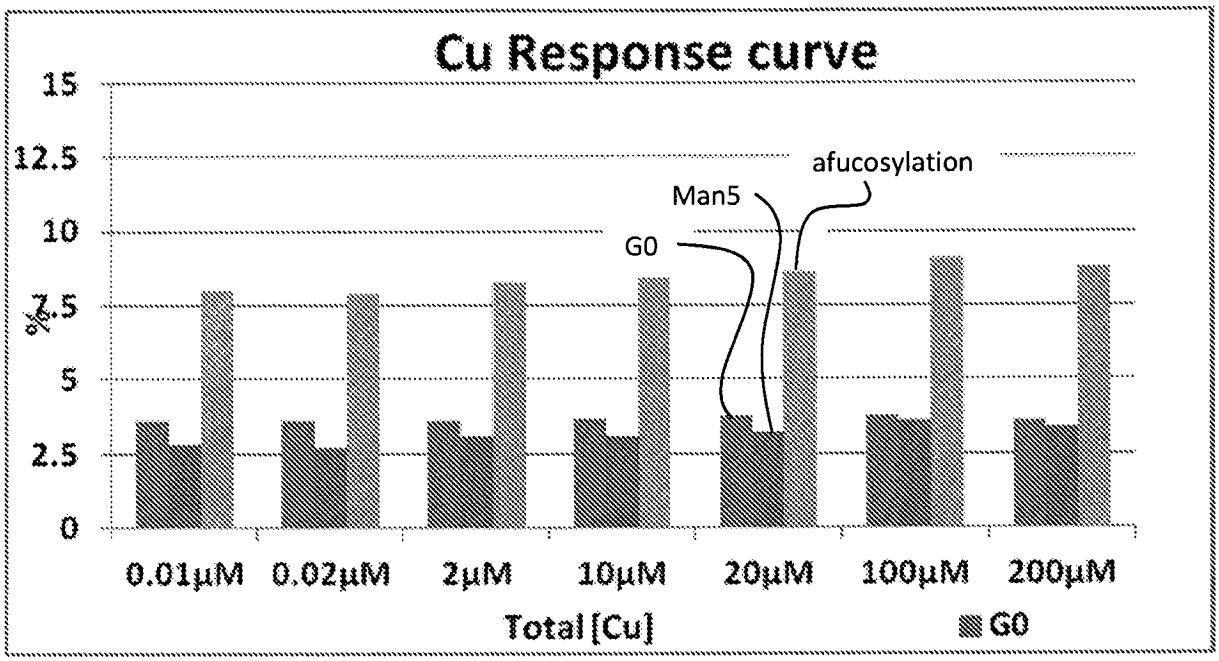

Cu Response curve
15
12.5
10
7.5
5
2.5
0
%
afucosylation
Man5
G0
0.01µM
0.02µM
2µM
10µM
20µM
100µM
200µM
Total [Cu]
G0

Fig. 13

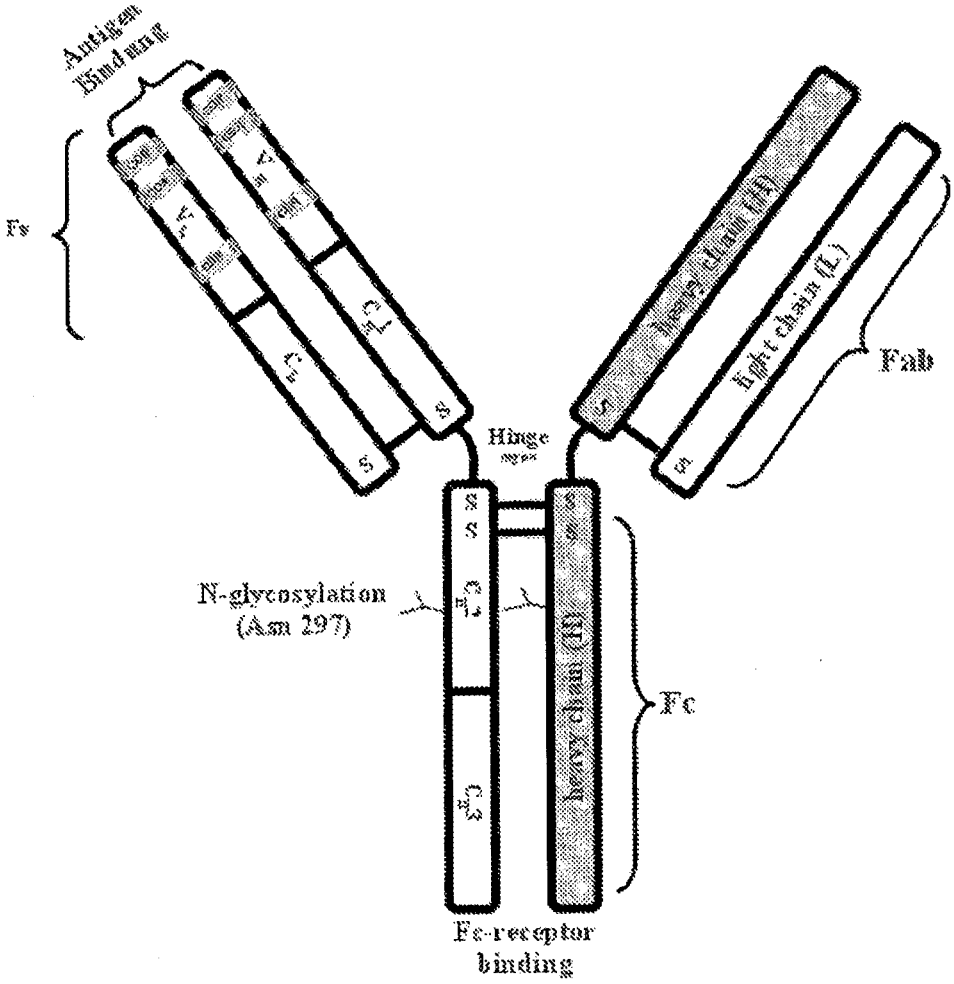
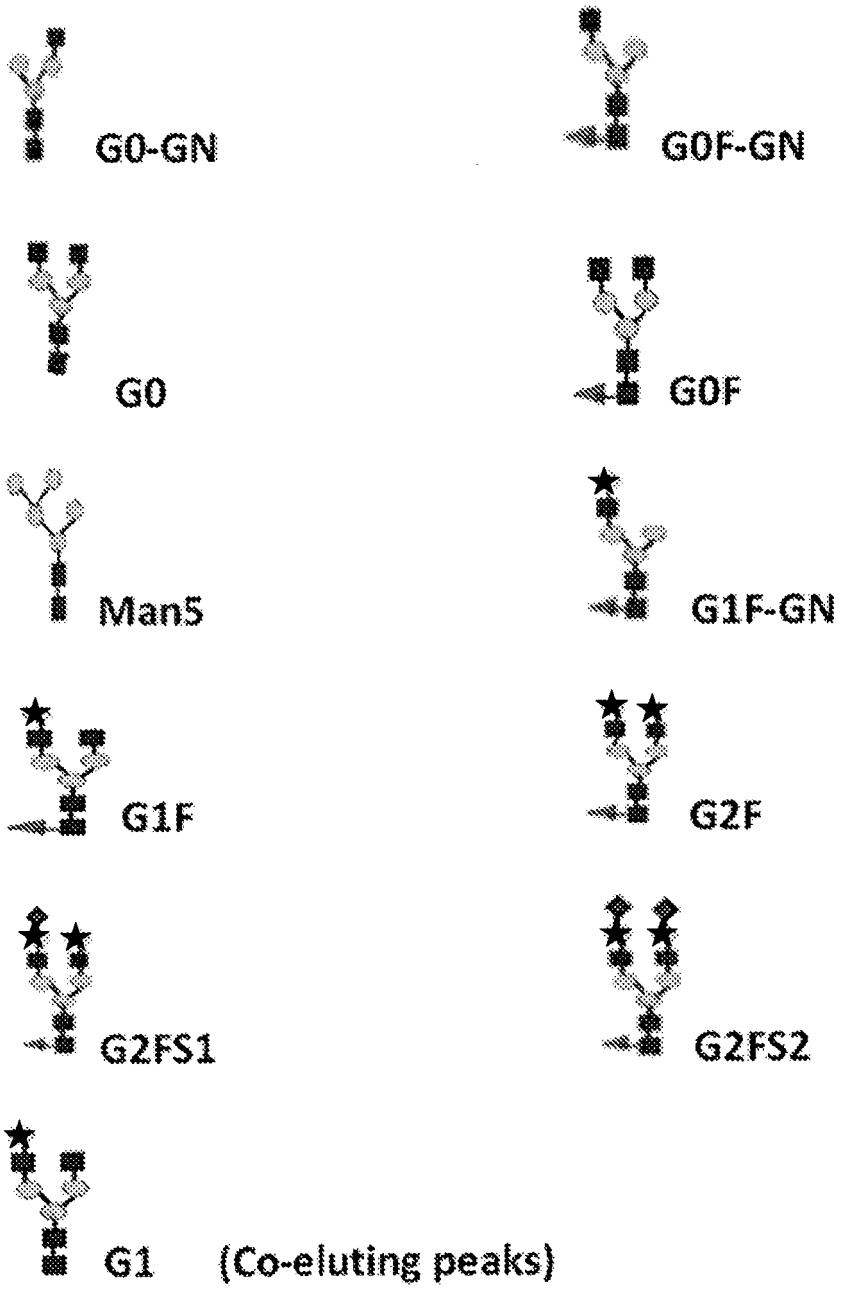

Antigen
Fv
Fab
Hinge
N-glycosylation
(Asn 297)
Fc
Fc-receptor
binding

Fig. 14



square – N-Acetyl Glucosamine, Circle – Mannose,
Star – Galactose, Triangle – Fucose, Diamond – Sialic acid

Fig. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/063348

A. CLASSIFICATION OF SUBJECT MATTER
C12N1/00,C12N5/00,C07K16/00,C07K14/00 Version=2014.01

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N,C07K

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

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

IPO Internal Database, Questel database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP2438185 A1 (BULIK DOROTA A) 11-04-2012 Abstract, Claims, Page 70, Page 122	1-2, 4-8, 13-16
Y	EP2438185 A1 (BULIK DOROTA A) 11-04-2012 Abstract, Claims, Page 70, Page 122	9-12,18-19
Y	Shitara K et al., "Experimental Therapeutics, Molecular Targets, and Chemical Biology: Defucosylated Chimeric Anti-CC Chemokine Receptor 4 IgG1 with Enhanced Antibody-Dependent Cellular Cytotoxicity Shows Potent Therapeutic Activity to T-Cell Leukemia and Lymphoma" Cancer Res March 15, 2004 64:2127-2133; doi:10.1158/0008-5472.CAN-03-2068 Abstract	9-11,18
Y	Montero E et al., "The inhibition of T cell proliferation in a mixed lymphocyte reaction by Itolizumab (T1h) is associated with reduction in pro inflammatory cytokines and CD6 internalization." The Journal of Immunology,	12,19

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17-11-2014	17-11-2014
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Anushri Kamble Telephone No. +91-1125300200

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/063348

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

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

1. ☒ Claims Nos.: 20, 22-24
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 22-24 relates to a method for treatment of the human or animal body by therapy, which does not require an international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

2. ☒ Claims Nos.: 21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The claim relates to the use of a glycoprotein according to any of claims 13-20 for the manufacture of a medicament for the treatment ofa human or animal patient.No meaningful search can be carried out since the technical features of the claim cannot be ascertained fromthe description and claims.

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

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/063348

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Meeting Abtracts, 2011, 186, 52.27 Abstract	
E,X	WO 2013114245 A1 (BHAVSAR KAUMIL) 08-08-2013 Abstract, Description	1-2, 4-8, 13-16
E,Y	WO 2013114245 A1 (BHAVSAR KAUMIL) 08-08-2013 Abstract, Description	9-12,18-19

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB2014/063348

Citation	Pub.Date	Family	Pub.Date
EP 2438185 A1	11-04-2012	WO 2010141855 A1	09-12-2010
		CA 2763164 A1	09-12-2010
		AU 2010256455 A1	19-01-2012
		US 2012277165 A1	01-11-2012

(19)中华人民共和国国家知识产权局

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C07K 16/00(2006.01)
C07K 14/00(2006.01)

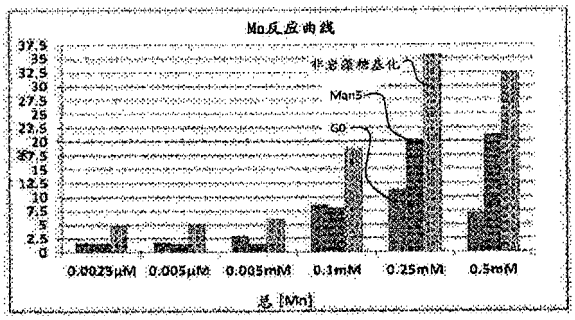
权利要求书2页 说明书14页 附图11页

(54)发明名称

用于控制蛋白质中的岩藻糖基化水平的方法

(57)摘要

本发明涉及一种用于控制、抑制或减少真核动物和/或真核动物的蛋白质表达系统中的蛋白质岩藻糖基化的方法或过程。所述方法包括在存在升高的锰或锰离子总浓度的情况下进行蛋白质表达和/或翻译后修饰。



CN 105392878 A

1. 一种用于修饰真核生物和 / 或真核生物的蛋白质表达系统中的岩藻糖基化的方法或过程，在所述方法或过程中控制培养基中的锰或锰离子的总浓度。

2. 根据权利要求 1 所述的方法或过程，所述方法或过程是减少岩藻糖基化的方法或过程，并且在所述方法或过程中，蛋白质表达和 / 或翻译后修饰是在存在升高的锰或锰离子的总浓度的情况下进行的。

3. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述升高的锰或锰离子的总浓度在≥ 0.05mM 至≤ 10mM 的范围内。

4. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述蛋白质表达和 / 或翻译后修饰是在选自昆虫细胞、真菌细胞、酵母细胞、原生动物细胞和 / 或哺乳动物细胞的蛋白质表达系统中进行的。

5. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述蛋白质是糖蛋白。

6. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述蛋白质是重组蛋白。

7. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述蛋白质是免疫配体。

8. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述免疫配体是选自下述的至少一种：鼠、嵌合、人源化、人的单克隆抗体或其衍生物、新抗体形式、由融合至靶结合部分例如受体片段的免疫球蛋白 Fc 区域组成的融合肽。

9. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，相较于在不存在升高的锰或锰离子浓度的情况下表达的免疫配体，所述免疫配体具有降低的岩藻糖基化程度。

10. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，相较于 (i) 在不存在升高的锰或锰离子浓度的情况下表达的或 (ii) 具有较高的岩藻糖基化程度的免疫配体，所述免疫配体显示增加的 ADCC 活性。

11. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述免疫配体靶向牵涉细胞介导免疫防御的一种或多种细胞表面抗原。

12. 根据前述权利要求中任一项所述的方法或过程，在所述方法中，所述免疫配体是 Itolizumab。

13. 一种糖蛋白，其通过根据前述权利要求中任一项所述的方法或过程产生。

14. 根据权利要求 13 所述的糖蛋白，所述糖蛋白是重组蛋白。

15. 根据权利要求 13 至 14 中任一项所述的糖蛋白，所述糖蛋白是免疫配体，优选地是抗体。

16. 根据权利要求 13 至 15 中任一项所述的糖蛋白，所述糖蛋白或其子结构域在其岩藻糖基化模式中具有减少的岩藻糖含量。

17. 根据权利要求 13 至 16 中任一项所述的糖蛋白，所述糖蛋白或其子结构域具有约 35％的非岩藻糖基化水平。

18. 根据权利要求 13 至 17 中任一项所述的糖蛋白，所述糖蛋白具有增加的 ADCC。

19. 根据权利要求 13 至 18 中任一项所述的糖蛋白，所述糖蛋白是 Itolizumab。

20. 根据权利要求 13 至 19 中任一项所述的糖蛋白，所述糖蛋白实现对于 CD25 和 CD4 呈阳性的细胞特别是 CD4+T 细胞在体外或体内的减少。

21. 根据权利要求 13 至 20 中任一项所述的糖蛋白用于制造治疗人或动物患者的药物的用途。

22. 根据权利要求 13 至 20 中任一项所述的糖蛋白用于治疗人或动物患者的用途。

23. 根据权利要求 21 至 22 中任一项所述的用途，其中所述人或动物患者患有选自以下的疾病或已被诊断为处于发生选自以下的疾病的风险中：

•肿瘤疾病，包括肿瘤、淋巴瘤及白血病，特别是 B 细胞慢性淋巴细胞性白血病 (B-CLL) 和 T 细胞白血病；

• 自身免疫疾病，包括类风湿性关节炎、银屑病、克罗恩氏病、红斑狼疮、舍格伦病；

• 神经退行性疾病，包括多发性硬化、帕金森氏病、阿尔茨海默病、亨廷顿病和 / 或肌萎缩侧索硬化；

• 感染性疾病。

24. 根据权利要求 21 至 23 中任一项的用途，其用于治疗或预防已进行移植的人或动物中的不良反应如移植物抗宿主病 (GVHD)。

用于控制蛋白质中的岩藻糖基化水平的方法

[0001] 相关申请的交叉参考

[0002] 本申请要求2013年7月23日向印度专利局提交的印度临时专利申请3262/CHE/2013及在2013年7月23日向印度专利局提交的印度临时专利申请3265/CHE/2013的权益及优先权。依据专利合作条约实施细则4.18，为了所有目的，2013年7月23日提交的所述申请的内容整体援引加入到本文中，包括加入未包含在本文中并在专利合作条约实施细则20.5(a)中提到的结论、权利要求书或附图的任何项目或部分。

技术领域

[0003] 本发明涉及用于控制蛋白质中的岩藻糖基化水平的方法。

[0004] 发明背景

[0005] 在真核生物表达系统中表达的蛋白质经历涉及糖基化的翻译后修饰过程。现今已被建立用于产生糖蛋白（如IgG及其它包含Fc区域的单克隆抗体）的真核生物表达系统将N-聚糖添加至多肽链。

[0006] 在IgG中，最重要的N-聚糖连接至两条CH2链的Asn297处（参见图14），其尤其包括N-乙酰基-神经氨酸（唾液酸）、N-乙酰基-葡糖胺、半乳糖、甘露糖及岩藻糖残基。

[0007] 这基本上适用于转基因植物表达系统以及适用于哺乳动物细胞系、昆虫细胞系等。在所有这些情况下，N-聚糖包含至少一个岩藻糖残基，其以α-3-糖苷或α-6-糖苷连接至与多肽链的Asn残基连接的N-乙酰基-葡糖胺残基。

[0008] 酵母表达系统易于产生富含甘露糖的高糖蛋白，其在治疗性抗体被施用至患者时常常导致非期望的免疫反应。杆状病毒转染的昆虫细胞系统因低糖基化而引起问题，其负面地影响治疗性抗体的效应子功能。此外，主要的缺点是感染性杆状病毒的催化性质，其使得用于完整IgG生产的范围(window)变窄。

[0009] ADCC是细胞介导免疫的机制，藉此免疫系统的效应细胞主动地裂解已被特异性抗体结合的靶细胞。它是作为体液免疫反应的一部分的抗体可以通过其作用来限制及遏制感染的多种机制中的一种。经典的ADCC介导的效应细胞是自然杀伤(NK)细胞；但单核细胞和嗜酸性粒细胞也可以介导ADCC。由于其依赖于先前的抗体反应，所以ADCC是适应性免疫反应的一部分。

[0010] 用于在靶细胞中引发ADCC的治疗性抗体需要Fc区域，以被所述效应细胞的Fcγ受体所识别。

[0011] 近来的研究已显示，相较于岩藻糖基化抗体，在其糖基化模式中具有减少量的岩藻糖的单克隆抗体显示出高得多的抗体依赖性细胞毒性(ADCC)活性。再次，岩藻糖残基的缺乏导致增加的ADCC的位置基本上是Asn297位置。低/无岩藻糖抗体的ADCC增加的机制似乎是由如此修饰的Fc区域针对FcγR(例如，FcγIIIa(CD16)，其是在人免疫效应细胞中针对ADCC的主要Fc受体）的亲和力增加所介导的(Shields等人，2002)。

[0012] 岩藻糖基化是牵涉糖蛋白或糖脂上的寡糖的最常见修饰中的一种。岩藻糖基化包括将岩藻糖残基附接至N-聚糖、O-聚糖及糖脂。O-岩藻糖基化（一种特殊类型的岩藻糖

基化）对于 Notch 信号转导非常重要。岩藻糖基化的调控机制是复杂的。许多种类的岩藻糖基转移酶、GDP- 岩藻糖合成途径及 GDP- 岩藻糖转运蛋白参与了岩藻糖基化的调控。

[0013] 已知糖基化影响治疗性单克隆抗体的效应子功能。在抗体的寡糖链中的不同糖残基中，岩藻糖是影响由产物诱导的抗体依赖性细胞毒性（ADCC）的关键糖类之一。

[0014] 通常使用细胞培养参数的操作来控制抗体的半乳糖苷化及唾液酸化。岩藻糖基化的控制主要通过使用 FUT8 敲除细胞和其它基因沉默模型通过细胞系工程改造来完成。

[0015] US20090208500 公开了通过操作 FUT8 敲除细胞来产生具有减少的岩藻糖和改善的 Fc 功能的抗体。

[0016] US7972810 公开了细胞培养方法和含锰培养基，其改善糖蛋白（包括红细胞生成素及其类似物或衍生物）的糖基化或唾液酸化。根据该公开内容，锰增加了唾液酸化和在 O- 连接及 N- 连接糖基化的情况下的位点占用（即较少的非糖基化产物），而且还增加末端半乳糖苷化。

[0017] 此外，可以由培养基渗透压来控制单克隆抗体的岩藻糖含量以用于高抗体依赖性细胞毒性（Konno 等人，2012）。

[0018] 然而，存在对于在所需细胞系中产生糖蛋白同时控制重组工程改造抗体的岩藻糖含量而无需每次经历在所选细胞系中产生 FUT8 基因敲除的费力过程的有效方法的需要。

[0019] 发明的实施方案

[0020] 这些目标通过使用根据本发明的独立权利要求的方法和手段得到满足。从属权利要求涉及优选实施方案。还应理解，由数值限定的取值范围应被理解为包含该限定值。

[0021] 发明概述

[0022] 在详细描述本发明之前，应理解，本发明不限制于所描述的装置的特定组成部分或所描述的方法的特定工艺步骤，因为这样的装置以及方法可发生变化。还应理解，本文使用的术语仅是为了描述具体实施方案的目的，并不意欲进行限制。必须注意，如在说明书及所附权利要求书中所使用的，除非上下文另外地清楚指明，单数形式“一个”、“一种”和“所述”包括单数和 / 或复数对象。此外，还应理解，在通过数值限定给出参数范围时，该范围被视为包括这些限定值。

[0023] 根据本发明的一个实施方案，提供了一种用于在真核生物和 / 或真核生物的蛋白质表达系统中修饰岩藻糖基化的方法或过程，在该方法或过程中，控制培养基中的锰或锰离子的总浓度。

[0024] 糖蛋白的岩藻糖基化是由岩藻糖基转移酶（FUT）完成的。这些酶是将 L- 岩藻糖从 GDP- 岩藻糖（鸟苷二磷酸岩藻糖）供体底物转移至受体底物的酶。受体底物可以是另一种糖，例如在 N- 连接糖基化的情况下将岩藻糖转移至核心 GlcNAc（N- 乙酰基葡糖胺）糖或是在由 O- 岩藻糖基转移酶产生的 O- 连接糖基化的情况下将岩藻糖转移至蛋白质。在哺乳动物中有不同的岩藻糖基转移酶，其中绝大多数位于高尔基体。最近已显示 O- 岩藻糖基转移酶定位于内质网（ER）。哺乳动物岩藻糖基转移酶的实例是 FUT1、FUT2、FUT3、FUT4、FUT5、FUT6、FUT7、FUT8、FUT9、FUT10 及 FUT11。

[0025] 尽管锰的作用还未被完全理解，锰是参与许多酶系统的必需微量元素。它在对于能量产生是必需的酶中用作辅因子，并且涉及葡萄糖的代谢作用、肝脏中的糖原储存、蛋白质消化作用以及胆固醇及脂肪酸的合成。它对于 DNA 和 RNA 分子的合成也是必需的。

[0026] 锰对于神经系统的生长及维护、骨和关节的发育及维护、雌性性激素及甲状腺激素的功能是必需的。超氧化物歧化酶（SOD，MnSOD）是一种在其结构中含有锰的抗氧化酶。

[0027] 在细胞外液体或真核生物中，锰实际上不存在，然而在哺乳动物中，锰的细胞内浓度是在0.010皮克/细胞至0.10皮克/细胞的范围内。

[0028] 然而，本发明人出人意料地发现，锰的浓度对于糖蛋白的岩藻糖基化水平具有直接效应。

[0029] 因此，本发明提供用于通过在过程中改变培养基及进料中的锰或锰离子的总浓度来修改糖基化蛋白质的岩藻糖含量。

[0030] 优选地，该方法或过程是降低岩藻糖基化的方法或过程。在这样的方法或过程中，蛋白质表达和/或翻译后修饰是在存在升高的锰或锰离子的总浓度的情况下进行的。

[0031] 出人意料地，本发明人发现，在这样的条件下，所表达的糖蛋白具有降低的岩藻糖基化水平。此外，他们发现，细胞生长、存活力及所产生的蛋白质的滴度未受锰或锰离子浓度升高的影响。

[0032] 此外，他们发现，糖基化模式的其它性质（即G0以及Man5）在存在升高的锰或锰离子的总浓度的情况下增加。

[0033] 如本文所使用，术语“岩藻糖基化水平”意指其中聚糖携带岩藻糖的糖蛋白的总量。同样地，术语“非岩藻糖基化水平”以及“非岩藻糖基化百分比”意指在其聚糖中没有岩藻糖的糖蛋白的百分比。

[0034] 在根据本发明的方法或过程的优选实施方案中，提供的升高的锰或锰离子浓度是在≥0.05mM至≤10mM的范围内。

[0035] 优选地，升高的锰或锰离子浓度为0.05；0.1；0.15；0.2；0.25；0.3；0.35；0.4；0.45；0.5；0.55；0.6；0.65；0.7；0.75；0.8；0.85；0.9；0.95；1；1.05；1.1；1.15；1.2；1.25；1.3；1.35；1.4；1.45；1.5；1.55；1.6；1.65；1.7；1.75；1.8；1.85；1.9；1.95；2；2.05；2.1；2.15；2.2；2.25；2.3；2.35；2.4；2.45；2.5；2.55；2.6；2.65；2.7；2.75；2.8；2.85；2.9；2.95；3；3.05；3.1；3.15；3.2；3.25；3.3；3.35；3.4；3.45；3.5；3.55；3.6；3.65；3.7；3.75；3.8；3.85；3.9；3.95；4；4.05；4.1；4.15；4.2；4.25；4.3；4.35；4.4；4.45；4.5；4.55；4.6；4.65；4.7；4.75；4.8；4.85；4.9；4.95；5；5.05；5.1；5.15；5.2；5.25；5.3；5.35；5.4；5.45；5.5；5.55；5.6；5.65；5.7；5.75；5.8；5.85；5.9；5.95；6；6.05；6.1；6.15；6.2；6.25；6.3；6.35；6.4；6.45；6.5；6.55；6.6；6.65；6.7；6.75；6.8；6.85；6.9；6.95；7；7.05；7.1；7.15；7.2；7.25；7.3；7.35；7.4；7.45；7.5；7.55；7.6；7.65；7.7；7.75；7.8；7.85；7.9；7.95；8；8.05；8.1；8.15；8.2；8.25；8.3；8.35；8.4；8.45；8.5；8.55；8.6；8.65；8.7；8.75；8.8；8.85；8.9；8.95；9；9.05；9.1；9.15；9.2；9.25；9.3；9.35；9.4；9.45；9.5；9.55；9.6；9.65；9.7；9.75；9.8；9.85；9.9；9.95； 或10mM。

[0036] 这些浓度意指在发生蛋白质表达和/或翻译后修饰的培养基中的总浓度。这意味着，例如，进料液（feed solutions）可以具有显著较高的锰或锰离子浓度。

[0037] 优选地，锰的浓度是通过将锰加至培养基和/或进料培养基来完成的。

[0038] 同样优选地，在蛋白质表达和/或翻译后修饰期间，锰浓度增加或降低。

[0039] 在根据本发明的方法或过程的优选实施方案中，提供的是，在选自以下的蛋白质

表达系统中进行蛋白质表达和/或翻译后修饰：

[0040] • 昆虫细胞；

[0041] • 真菌细胞；

[0042] • 酵母细胞；

[0043] • 原生动物细胞；和/或

[0044] • 哺乳动物细胞。

[0045] 优选地，哺乳动物细胞选自鼠细胞（例如，NS0）、仓鼠细胞（例如，CHO或BHK）和/或人类细胞（例如，PER.C6）。

[0046] 优选地，蛋白质是糖蛋白。更优选地，蛋白质是重组蛋白。

[0047] 在根据本发明的方法或过程的优选实施方案中，提供的是，蛋白质是免疫配体。

[0048] 本文所使用的术语“免疫配体”意指具有以足够程度的灵敏度和/或特异性与另一生物学实体结合的能力的实体。

[0049] 在根据本发明的方法或过程的另一个优选实施方案中，提供的是，免疫配体是选自以下的至少一种：

[0050] • 单克隆抗体（鼠、嵌合、人源化、人）或其衍生物

[0051] • 新的抗体形式

[0052] • 由融合至靶结合部分（例如受体片段）的免疫球蛋白Fc区域组成的融合肽。

[0053] 优选地，以上列出的免疫配体包含能够被糖基化和/或与Fc受体（例如FcγRI(CD64)，FcγRIIA(CD32)，FcγRIIB(CD32)，FcγRIIIA(CD16a)，FcγRIIIB(CD16b)）结合的Fc区域或另一种结构域。

[0054] 如本文所使用的，术语“单克隆抗体(mAb)”应意指具有同质抗体群体（即，由完整的免疫球蛋白或其片段或衍生物组成的同质群体）的抗体组成物。特别优选的，这样的抗体选自IgG、IgD、IgE、IgA和/或IgM、或其片段或衍生物。

[0055] 如本文所使用，术语“衍生物”应意指与一般抗体概念结构上不同但仍具有某些结构关系的蛋白质构建体。

[0056] 用于产生和/或选择嵌合、人源化和/或人mAb的方法是本领域已知的。例如，Genentech的US6331415描述嵌合抗体的产生，而Medical Research Council的US6548640描述CDR移植技术，以及Celltech的US5859205描述人源化抗体的产生。除其它以外，体外抗体文库公开于MorphoSys的US6300064以及MRC/Scripps/Stratagene的US6248516中。噬菌体展示技术例如被公开于Dyax的US5223409中。转基因哺乳动物平台例如被描述于TaconicArtemis的US200302048621中。

[0057] 术语“新抗体形式”包含例如双特异性或三特异性抗体构建体、双体抗体、骆驼科动物抗体、域抗体、具有由scFvs组成的两条链的双价同二聚体、IgAs（通过J链和分泌性成分连接的两个IgG结构）、鲨鱼抗体、由新世界灵长类动物框架加上非新世界灵长类动物CDR组成的抗体、包含CH3+VL+VH的二聚化构建体以及抗体缀合物（例如，连接到毒素、细胞因子、放射性同位素或标记的抗体或片段或衍生物）。然而此列表不是限制性的。

[0058] 此外，该术语也包括免疫毒素（即，由抗体或其片段组成的杂二聚体分子），以及细胞毒性、放射性及细胞凋亡因子。这样的形式类型例如已由Philogen开发（例如，与人TNF融合的抗-EDB人抗体L19）或是由与细胞毒性的Mertansine(DM1)连接的曲妥珠单抗

组成的 Trastuzumab emtansine(T-DM1)。

[0059] 术语“融合肽”或“融合蛋白”涉及例如由免疫球蛋白 Fc 部分加上靶结合部分组成的蛋白质（所谓的 -cept 分子）。

[0060] 在根据本发明的方法或过程的另一个优选实施方案中，提供的是，相较于在不存在升高的锰或锰离子浓度的情况下表达的免疫配体，该免疫配体具有减少程度的岩藻糖基化。

[0061] 优选地，根据本领域的方法来测定岩藻糖基化程度。除其它以外，这样的方法包括用肽 -N- 糖苷酶 F(PNGase F) 消化以将抗体去糖基化（更多细节参见图 1 的描述），和随后收集分离出的聚糖。将收集到的聚糖用邻氨基苯甲酸标记，并且随后藉由 NP HPLC 进行分析。该方法的全部细节公开于 Anumula(2012)，其内容通过引用并入本文。

[0062] 术语“不存在升高的锰或锰离子浓度”是指在该过程期间或在该过程的准备中，没有出于自愿地引入锰或锰离子。这并未排除仍可存在微量的锰，其天然存在于介质（如水）中。

[0063] 在根据本发明的方法或过程的优选实施方案中，提供的是，相较于 (i) 在不存在升高的锰或锰离子浓度的情况下表达的或 (ii) 具有较高的岩藻糖基化程度的免疫配体，该免疫配体显示增加的 ADCC 活性。

[0064] 术语“ADCC”涉及细胞介导免疫防御的机制，藉此免疫系统的效应细胞主动地裂解靶细胞，其膜表面抗原已被特异性抗体结合。它是作为体液免疫反应的一部分的抗体通过其可以用来限制及遏制感染的多种机制中的一种。经典的 ADCC 是由自然杀伤 (NK) 细胞介导的；巨噬细胞、嗜中性粒细胞及嗜酸性粒细胞也可以介导 ADCC。由于其依赖于先前的抗体反应，所以 ADCC 是适应性免疫反应的一部分。

[0065] 优选地，通过根据本领域的方法来测定 ADCC 活性。除其它以外，这样的方法包括如图 3 显示的细胞毒性测定法。

[0066] 其它合适的测定法包括铬 -51[Cr51] 释放测定法、铕 [Eu] 释放测定法及硫 -35[S35] 释放测定法。通常，表达某一表面暴露抗原的被标记的靶细胞系与特异于该抗原的抗体一起温育。在洗涤后，表达 Fc 受体 CD16 的效应细胞与抗体标记的靶细胞共温育。随后，以闪烁计数器或分光光度法通过细胞内标记的释放来测量靶细胞裂解。耦合的生物发光方法 aCella TOX 现在普遍地用于 ADCC 及其它细胞毒性评估。由于此技术测量天然存在于靶细胞中的酶的释放，不需要标记步骤并且不使用放射性试剂。

[0067] 优选地，免疫配体靶向牵涉细胞介导免疫防御的一种或多种细胞表面抗原。

[0068] 优选地，所述细胞表面抗原选自亲环蛋白 C、补体因子 I、CD6、CD5、牛 WC-1 和 M130。

[0069] CD6 是主要由人 T 细胞及 B 细胞的子集以及由一些 B 细胞慢性淋巴细胞性白血病和神经元表达的一种重要的细胞表面蛋白 (Aruffo 等人 1991，Kantoun 等人 1981，Mayer 等人 1990)。CD6 是蛋白质大家族的一员，特征在于具有与 I 型巨噬细胞的清道夫受体的富含半胱氨酸结构域 (SRCR) 同源的至少一个结构域 (Matsumoto 等人 1991 以及 Resnick 等人 1994)。此家族的其它成员包括 CD5(Jones 等人，1986)、亲环蛋白 C(Friedman 等人 1993)、结合活化的补体蛋白 C3b 及 C4b 的补体因子 I(Goldberger 等人，J. Biol. Chem. 1987，262:10065)、由 . τ./. δ . T 细胞表达的牛 WC-1(Wijingaard 等人 1992) 以及 M130(Law 等人 1993)，其是巨噬细胞活化标志物。

[0070] 其它优选的表面抗原包括 CD20、EGFR、HER2/neu 以及膜结合的 TNF。

[0071] 在根据本发明的方法或过程的优选实施方案中，提供的免疫配体是 Itolizumab。

[0072] Itolizumab(INN，商品名 **Alzumab ®** ）是由 Biocon 开发的一种“全新”人源化 IgG1 单克隆抗体。它选择性地靶向 CD6，其是牵涉 T 细胞的共同刺激、附着及成熟的泛 T 细胞 (pan T cell) 标志物。Itolizumab 通过与 CD6 结合下调 T 细胞活化，导致促炎症细胞因子合成的减少并且可能通过减少 T 细胞在炎症位置的渗入作用而发挥重要的作用。相较于安慰剂，itolizumab 的双盲的、安慰剂对照的、III 期治疗 - 斑块研究 (phase III treat - Plaq study) 在治疗具有中度至严重银屑病的患者 12 周后，成功地满足了 PASI-75 分（银屑病面积和严重指数）得分的显著改善的预先指定主要终点。Biocon 在 2013 年 1 月从印度药品管理总局 (DCGI) 收到了药品销售许可，并且在 2013 年 8 月在印度境内开始销售 (Jayaraman，2013)。

[0073] 从小鼠衍生的 NS0 细胞系（本文称做“T1h”）并且还从中国仓鼠卵巢 (CHO) 细胞系（本文称做“Bmab-600”）产生 Itolizumab。因为翻译后修饰，特别是非岩藻糖基化模式随着细胞系和培养条件而变化，Bmab-600 及 T1h 的 Fc 部分以不同的亲和力与 Fc γ RIIIa 结合。

[0074] 可以例如从小鼠衍生的 NS0 细胞系（本文称做“T1h”）并且还从中国仓鼠卵巢 (CHO) 细胞系（本文称做“Bmab-600”）产生 Itolizumab。因为翻译后修饰，特别是非岩藻糖基化模式随着细胞系和培养条件而变化，Bmab-600 及 T1h 的 Fc 部分以不同的亲和力与 Fc γ RIIIa 结合。

[0075] 根据本发明的另一方面，提供了一种糖蛋白，所述糖蛋白用根据本发明的任何方法或过程的方法或过程来产生。

[0076] 优选地，所述糖蛋白是重组蛋白。更优选地，所述糖蛋白是免疫配体，优选是抗体。特别优选的是，所述糖蛋白在其糖基化模式上具有减少的岩藻糖含量。

[0077] 优选地，糖蛋白或其子结构域（如 Fc 区域）具有约 35％的非岩藻糖基化水平。

[0078] 在优选实施方案中，提供的是，糖蛋白具有增加的 ADCC。优选地，所述糖蛋白是 Itolizumab。

[0079] 在另一个优选实施方案中，提供的是，糖蛋白实现对于 CD25 和 CD4 呈阳性的细胞特别是 CD4+T 细胞在体外或体内的减少。

[0080] 本发明人出人意料地显示，根据本发明的抗 CD6 抗体的使用导致对于表面抗原 CD25 和 CD4（参见图 5b 及描述）呈成阳性的细胞特别是 CD4+T 细胞的增殖减少。

[0081] 如本文所使用的术语“细胞的减少”意指 (i) 增殖的抑制、(ii) 耗尽、(iii) 细胞凋亡的诱发或 (iv) 导致这样的细胞的减少的其它机制。

[0082] 根据本发明的另一方面，提供了如上所示的糖蛋白用于制造治疗人或动物患者的药物的用途。同样地，提供了如上所示的糖蛋白用于治疗人或动物患者的用途。

[0083] 在这样的用途的优选实施方案中，人或动物患者患有选自以下的疾病或已被诊断为处于发生选自以下的疾病的风险中：

[0084] • 肿瘤疾病，包括肿瘤、淋巴瘤及白血病，特别是 B 细胞慢性淋巴细胞性白血病 (B-CLL)，特别是 T 细胞白血病

[0085] • 自身免疫疾病，包括类风湿性关节炎、银屑病、克罗恩氏病、红斑狼疮和 / 或舍格

伦病

[0086] • 神经退行性疾病，包括多发性硬化和 / 或帕金森氏病、阿尔茨海默病、亨廷顿病和 / 或肌萎缩侧索硬化，和 / 或

[0087] • 感染性疾病

[0088] 优选地，这样的用途涉及治疗或预防已进行移植的人或动物中的不良反应如移植物抗宿主病 (GVHD)。这样的移植包括器官移植以及骨髓移植。

[0089] 实验和附图 / 实施例

[0090] 虽然已在附图以及前文描述中详细说明及描述本发明，但这样的说明及描述应被认为是示例性或范例性的且不是限制性的；本发明并不受限于所公开的实施方案。本领域普通技术人员在实施请求保护的发明中可以通过研读附图、公开内容及所附权利要求书而理解并实现对公开的实施方案的其它变化。在权利要求书中，单词“包含”未排除其它元素或步骤，而且不定冠词“一个”或“一种”并未排除复数。仅某些措施记载于互相不同的从属权利要求中的事实并非表明不能使用这些措施的组合以处于优势。权利要求中的任何参考记号不应被解释为限制范围。

[0091] 图 1：用具有 Fc 区域的抗体进行的去糖基化实验的结果。

[0092] 将抗 CD 抗体 Itolizumab（也称做 T1h）用去糖基化缓冲液 (50mM Tris，1mM CaCl2，pH = 8.1) 以对于 Itolizumab(5mg/ml)1:1 的比例进行温育，随后用肽 -N- 糖苷酶 F(PNGase) 酶（对于 1mg 抗体为 10U) 温育 24 小时。

[0093] 在 37℃下温育 24 小时后，在样品中加入相等体积的 T1h 缓冲液（组氨酸海藻糖缓冲液）并且在 4℃、4000rpm 于 centricon 管 (50kD 截断过滤器）中离心 15 分钟。将残留体积再次以相等体积的 T1h 缓冲液重新悬浮，并在 4℃、4000rpm 下离心 15 分钟。将去糖基化的 Ab 储存于最终的储存管中，并通过 Nano drop 估计浓度。通过 CE-SDS（毛细管电泳）确认去糖基化。随后进行荧光活化细胞分选仪 (FACS) 分析。简言之，用抗 CD6 抗体 T1h 或如上所述产生的去糖基化的 T1h 抗体标记 HUT78 细胞 (T 细胞系）。

[0094] 随后使用 FITC 标记的抗 Fc 的第二抗体，观察到信号。图 1 显示 Th1 的 Fc 区域的去糖基化并未损及其与表达 CD6 的细胞系结合的能力。这些结果进一步由等离子体共振实验确认。

[0095] 图 2：使用具有 Fc 区域的抗体进行的去糖基化实验的结果。

[0096] 具体地，如上文所讨论的，将抗 CD 抗体 Itolizumab 去糖基化。其抑制活化的 T 细胞增殖的能力随后在合适的增殖测定法中与未经修饰的 T1h 的能力进行比较。使用尼妥珠单抗作为阴性对照，尼妥珠单抗是具有与 Itolizumab 相同的 IgG 骨架但与 EGFR 结合的抗体。

[0097] 简言之，使用碳酸氢盐缓冲液 (pH 9.5) 将抗体以 0 至 1 μg/ml 范围的浓度涂布在无菌的 96 孔板中过夜。在洗涤后，将来自正常健康志愿者的纯化淋巴细胞加至板中。加入从 80 至 1 μg/ml 的 Itolizumab，并且将培养物温育 4 天。加入阿拉玛蓝以测量增殖。相对于未受刺激的细胞对照来计算倍数差异。使用同种型尼妥珠单抗抗体作为对照。附着在板上的抗 CD3（所使用的抗 CD3 是在古巴分子免疫学中心制造的 OKT3 克隆）刺激来自正常健康的志愿者的幼稚 T 细胞（来自在 Ficoll 密度梯度上纯化的人供体的外周血单核细胞 (PBMC)）的增殖。

[0098] 尼妥珠单抗(80μg/ml)不显示任何的T-细胞增殖抑制,与未受刺激的细胞相比,导致了大约2.75倍的细胞增加,而天然的T1h显示T-细胞增殖的抑制(在80μg/ml至1.25μg/ml的范围为35至20%抑制)。与此相反,去糖基化的T1h的影响与尼妥珠单抗的影响类似。这意味着,在去糖基化后,抗体失去其抑制T细胞增殖的能力。

[0099] 图3:比较天然抗体和去糖基化抗体的细胞毒性测定法的结果

[0100] 将冷冻的PBMC于具有10% FBS的RPMI1640培养基中在IL-2(浓度2.5ng/mL)的存在下解冻,并于37℃、5% CO_2培养箱中温育过夜。第二天,将细胞重新悬浮于没有IL-2的培养基中并温育4至5小时。将12,000个Hut-78细胞/50μL加入96孔板中的每一孔。按照模板加入50μL的3X浓缩药物(10微克/毫升的天然的T1h、去糖基化的T1h或抗CD3)并于37℃、5% CO_2培养箱中温育2小时。将PBMC重新悬浮于测定培养基中并加入240,000个PBMC/50μL/孔,以获得1:20的靶对效应物的比例。将板于37℃、5% CO_2培养箱中温育22小时。将50μL的Cyto Tox-Glo加至板并且于室温温育30分钟。使用Spectramax来读取板的发光,以测定细胞毒性。

[0101] 虽然天然的T1h相较于抗CD3(其为靶向T细胞的部分耗尽抗体)显示轻微的但统计上一致性的抗体依赖性细胞毒性(ADCC)活性,但该ADCC活性在分子的去糖基化后显著地降低,表明了T1h的效应子功能。使用Itolizumab的Fab2片段也可以可与去糖基化分子相当地降低ADCC活性。

[0102] 图4:比较天然的抗体和去糖基化的抗体的混合性淋巴细胞反应(MLR)实验的结果。

[0103] PBMC的制备:从健康供体收集30ml的血液。通过标准FICOLL密度梯度离心来分离PBMC。

[0104] 单核细胞耗尽及建立

[0105] 树突细胞(DC)衍生测定:将这些细胞于 CO_2培养箱中温育2小时。使单核细胞附着在塑料表面上。随后将未附着的细胞(PBL)从培养瓶移除。用1XPBS洗涤所有的培养瓶一次。将20ml的DC培养基(制成50ml贮存液,在50ml的测定培养基中的10μl粒细胞巨噬细胞集落刺激因子(GMCSF)和5μl的IL-4)加至每一培养瓶。将培养瓶保持在 CO_2培养箱中6天。

[0106] 对生长中的树突细胞进行LPS处理:在第6天,将具有脂多糖(LPS)的DC培养基加入每一培养瓶(在培养瓶中的LPS最终浓度为4ug/ml)并且放回CO2培养箱中40至48小时。

[0107] DC的制备:在LPS处理后,从两个培养瓶收集细胞悬浮物(DC)。用1XPBS洗涤每一培养瓶一次。以1500rpm、5分钟转下细胞悬浮物,并且于3ml培养基中重建。计数LPS处理过的DC,并且按照测定要求在培养基中重建。

[0108] PBL的制备:采用如前文所述的相同方案,在从另一健康个体收集血液后,进行Ficoll分离。在单核细胞耗尽之后,收集未附着的外周血淋巴细胞(PBL)并以1500rpm、5分钟转下并在5ml培养基中重建。计数PBL并重建至 $1.0x10^6$个细胞/毫升。

[0109] 对树突细胞(DC)进行SEB处理:葡萄球菌肠毒素B(SEB)贮存液浓度为1mg/ml。从贮存液中,将3μl的SEB溶解于3ml的培养基,得到1mg/ml的SEB工作溶液。按照标准方案,用0.6ug的SEB处理 $0.06x10^6$个DC。制得 $0.1x10^6$个细胞/毫升的贮存液(LPS处

理过的成熟 DC)。由此,将 600 μ l 的细胞悬浮物溶解于 2.4ml 的测定培养基(细胞悬浮物的总体积为包含 0.02x10^{6}个细胞 / 毫升的 3ml)中。将其以 1500rpm、5 分钟旋转,并且将 600ml 的 SEB(1ug/ml)加至沉淀物。将其在 37℃下于 CO2 培养箱内温育 20 分钟。温育后将过量的培养基(2ml)加至试管内,并且以 1500rpm 洗涤 5 分钟。丢弃上清液,并且再次用 3ml 的培养基洗涤细胞。最后将沉淀物溶解于 3ml 的测定培养基。

[0110] 对 PBL 进行丝裂霉素 C 处理:从 1mg/ml 的丝裂霉素贮存液制得 25 μ g/ml 的丝裂霉素溶液。在 37℃下于 CO_2培养箱内用 500 μ l 的 25 μ g/ml 丝裂霉素对 0.5x10 ^{6}个 PBL 处理 30 分钟。温育后,向其加入过量的培养基(2ml),并在 1500rpm 下洗涤细胞 5 分钟。丢弃上清液,并且再次用 3ml 的培养基洗涤细胞。

[0111] MLR 测定 - 增殖的抑制:以 DC:PBL = 1:50 的比例进行 MLR 测定。使用的阴性对照是尼妥珠单抗。将天然的 T1h 针对其缺少完整功能的 Fc 区域的 Fab2 形式进行测试。6 天后使用 Bio-Tek Synergy HT Gen5 读板器利用阿拉玛蓝来读取板。

[0112] 完整的抗体可以抑制在此反应中诱发的 T 细胞增殖,而具有不同特异性的阴性对照尼妥珠单抗则不能。没有 Fc 区域的 T1h 也无法抑制 T 细胞增殖,表明在此测定中糖基化的 Fc 区域和 Fab 对 T1h 的抑制能力是关键性的。在使用去糖基化的 T1h 中也观察到类似的效应,由此确认 T1h 的效应子功能需要糖基化。

[0113] 图 5a:比较不同免疫调节剂的另一混合淋巴细胞反应(MLR)实验的结果

[0114] 实验方案与图 4 相同。其为混合淋巴细胞反应。除了 4 倍浓度的天然 T1h 外,还使用其它免疫抑制剂及免疫调节剂,亦即吡美莫司(Pim)、阿巴西普(Aba)及达利珠单抗(Dac)被包括作为用于测定的阳性对照。尼妥珠单抗(hR3)用作阴性对照。

[0115] 事实证明,相较于与人 EGFR 结合的特同种型抗体尼妥珠单抗,T1h 能够降低混合性淋巴细胞反应中诱导的 T 细胞增殖。由 T1h 诱导的倍数降低可与由阿巴西普(CTLA4-IgG1Fc)、达利珠单抗(抗 CD25)及吡美莫司(小分子,IL2 抑制剂)诱导的倍数降低相当。

[0116] 图 5b:显示于图 5a 中的实验的分析。

[0117] 该分析涉及 144 小时(6 天)混合性淋巴细胞反应后来自培养物的细胞。B--、B++、B+- 和 B-+ 为四个象限。这里,在 6 天后评估 MLR 的培养物中的细胞。虽然与其它抗体相较,T1h 有好的抑制能力,但 T1h 的途径是不同的,因为在这里与使用其它分子的实验不同,在 CD4/CD25 活化的 T 细胞群中存在显著减少。T1h 显示出 CD25+、CD4+ 以及 CD4+T 细胞的减少。这表明 T 细胞子集的选择性耗尽。因此,虽然如图 5a 所显示,在 MLR 中通过 T1h 的抑制可与达利珠单抗、阿巴西普及吡美莫司的抑制相当,但仅 T1h 显示 CD25+、CD4+ 以及 CD4+T 细胞的减少。

[0118] 图 6:比较天然抗体和去糖基化抗体的细胞毒性测定的结果。

[0119] 图 3 中的相同测定被用于(与阳性对照(抗 -CD3)相比较)评估具有不同非岩藻糖基化含量的抗体。显示的数据是来自 n = 4 的独立实验的汇编。

[0120] 如本文其它处所描述地进行非岩藻糖基化(参见,例如图 11 的描述)。Itolizumab 的 Fc 区域的非岩藻糖基化增加显示由该抗体相对于阳性对照抗体(抗人 CD3)展示的 ADCC 活性的线性增加。这证实了通过仅增加非岩藻糖基化的 Fc 聚糖含量使 ItoHzumab 更具细胞毒性的能力。例如,为了将 ADCC 从相对于抗 CD3 的活性的 20%增强到高于 40%,抗体中

的非岩藻糖基化含量应大于 10%。

[0121] 如于下文讨论的 biacore 数据所示，这样的增加可通过与 Fc γ RIII 更好的结合而引起（其中 Bmab 600 以相较于 T1h 的更好亲和力来结合）。因此在抗体中增加非岩藻糖基化种类可引起与 Fc γ RIII 的更好结合，并且这转变为更好的 ADCC 功能活性。

[0122] 图 7 :比较 T1h 和利妥昔单抗的 CDC 测定的结果

[0123] 使用人 T 细胞淋巴瘤细胞系 Hut78（ **ATCC®** TIB-161™）来评估 T1h 的 CDC 活性。将 $1x10^4$个细胞与 10 μg/mL、1 μg/mL 和 0.01 μg/mL 的各药物稀释液在 37℃、5% CO_2 培养箱中温育 20 分钟。以 1:10 的最终浓度加入合并的正常人血清，并在 37℃将细胞温育 2 小时。加入 **AlamarBlue®** (Invitrogen) 并在 37℃将细胞温育 20 至 22 小时。染料被细胞的摄取和随后它的降低被读取为在 530/590nm 处的荧光。

[0124] 在测定中使用利妥昔单抗（一种靶向 B 细胞系 (Daudi) 上的 CD20 受体并引起补体依赖性细胞毒性 (CDC) 的抗 CD20）作为阳性对照，以显示导致 CDC 的血清组分是完整的。

[0125] T1h 未显示 CDC 活性。Itolizumab 的非岩藻糖基化种类的增加没有增加该分子的 CDC 活性，从而得出结论为只有 ADCC 效应子功能随着非岩藻糖基化的增加而增强。

[0126] 表 1 :图 6 和 7 所示的测定中使用的差别非岩藻糖基化 T1h 样品的聚糖特征谱。

[0127]

批次	G0-GN	G0f-GN	G0	G0f	Man5	G1f-GN,G1	G1f	Man6,(G1f-GN)S1	G1FS1，具有1G，总合物S1的三触角复合物	G2f	G2fs1，具有小型总合物	G2fS2	其它混合种类	总的非岩藻糖基化种类
T1h范围	0.1-0.5	0.8-2.2	0.1-0.3	23.5-36.7	1.2-3.7	1.2-3.4	37.9-53.8	0.6-1.4	1.6-2.6	6.2-12.2	0.5-13.6	1.5-3.8	0.5-1.3	3.55
1185/12/03/250	0.7	2.2	5.1	45.1	3.0	3.4	31.3	1.3	1.0	4.2	1.9	0.2	0.7	10.1
1185/12/03/270	0.5	1.5	4.3	42.7	2.6	3.5	34.4	1.3	1.0	5.0	2.1	0.3	0.8	8.7
1185/12/03/340	0.5	1.3	4	45.1	6.8	2.9	25.2	1.8	0.9	4.2	1.9	0.7	0.9	13.1
1185/12/03/310	0.6	1.5	4.5	42.4	7.3	3.7	29.1	2.1	1.0	4.0	2.3	0.3	1.1	14.5
1185/11/01/350	0.7	1.4	6.5	48.7	4.8	3.5	28.2	1.4	0.8	3.4	1.3	0.6	0.7	13.4

[0128] 用标准方法进行糖基化模式的分析。简言之，用肽 -N- 糖苷酶 F(PNGase F) 消化抗体，以使抗体去糖基化（对于更多细节参见图 1 的描述），并且收集分离的聚糖。将收集到的聚糖用邻氨基苯甲酸标记，并且随后藉由 NP HPLC 进行分析。该方法的全部细节公开于 Anumula(2012)，其内容通过引用并入本文。

[0129] 在此表中，使用了以下的缩写 :G0 = 没有半乳糖，G1 = 1 个末端半乳糖残基，G2 = 2 个末端半乳糖残基，GN = N- 乙酰基葡糖胺或 GlcNac，F = 岩藻糖，Man5 = 5 个甘露糖残基，Man6 = 6 个甘露糖残基以及 S = 唾液酸。

[0130] 在图 15 中提供了在本文所示的实验过程中所测定的糖基化模式的说明以及所使用的命名法。

[0131] 图 8 至图 10 :用等离子体共振检测 T1h 对 Fc γ RIIIa 的结合曲线。

[0132] BIAcore 是一种分析装置，其检测接近传感器表面的基于表面等离子体共振的折射率变化的差异。测定抗体针对 Fc 受体配体的亲和力常数的此方法已被广泛使用。为了检测相互作用，将一个分子（配体）固定在传感器表面上。将它的结合配偶体（分析物）通过流动池以及在连续的流动下注入到水溶液（样品缓冲液）中。当分析物与配体结合时，蛋白质在表面上的累积导致了折射率的增加，其对时间作图以产生感应图。从感应图的分析测定缔合常数 (K_a)、解离速率常数 (K_d) 及平衡解离常数 (K_D)。

[0133] FcγRIIIa被认为是一种中间型亲和受体。它可以变化地结合单体IgG并且似乎与较低亲和力Fcγ受体相比具有对IgG的高亲和力。它们在NK细胞和血液细胞的单核细胞上表达。

[0134] 因为翻译后修饰，特别是非岩藻糖基化模式随细胞系和培养条件而变化，Bmab-600和T1h的Fc部分以不同的亲和力结合至FcγRIIIa。我们在Biacore仪器中评估这两种产物针对FcγRIIIa的结合亲和力。相较于T1h，Bmab-600的结合亲和力结果显示与FcγRIIIa受体结合的更高亲和力。在固定有FcγRIIIa受体的表面上分析以下样品：

[0135] 1. T1h抗体

[0136] 2. Bmab-600抗体

[0137] 3. 去糖基化T1h抗体

[0138] 每一样品分析两次并报告平均K_D(μM)值并且彼此比较。图8显示T1h抗体对FcγRIIIa的结合曲线，图9显示Bmab-600抗体对FcγRIIIa的结合曲线，以及图10显示去糖基化T1h抗体对FcγRIIIa的结合曲线。

[0139] 该方法是灵敏的并且能够区分固有地存在于Bmab-600和T1h的差异非岩藻糖基化样品中的非岩藻糖基化差异之间的不同。该数据还显示，随着非岩藻糖基化水平增加，FcγRIIIa结合亲和力值成比例地降低（意味更高的亲和力）。还通过在没有观察到结合相互作用时分析T1h的去糖基化样品证实该方法的特异性。

[0140] 表2：T1h抗体对FcγRIIIa的动力学值（参见图8）

[0141]

样品	Ka (1/Ms)	kd (1/s)	K_D (M)	K_D (μM)	平均 K_D (μM)
T1h(NS0)	1.52E+04	5.65E-03	3.72E-07	0.372	0.440
	1.16E+04	5.88E-03	5.08E-07	0.508	

[0142] 表3：Bmab600抗体对FcγRIIIa的速率常数值（参见图9）

[0143]

样品	ka (1/Ms)	kd (1/s)	K_D (M)	K_D(μM)	平均 K_D(μM)
Bmab-600(CHO)	2.46E+04	4.76E-03	1.93E-07	0.193	0.200
	2.78E+04	5.73E-03	2.07E-07	0.207	

[0144] 表4：去糖基化T1h抗体对FcγRIIIa的速率常数值（参见图10）

[0145]

样品	ka (1/Ms)	kd (1/s)	K_D (M)	K_D (μM)	平均 K_D (μM)
T1h (NS0)	阴性相互作用				

[0146] 表 5:Bmab-600 和 T1h 抗体对 Fc γ RIIIa 的差异非岩藻糖基化样品的速率常数值(参见图 10)

[0147]

样品	非岩藻糖基化%	ka (1/Ms)	kd (1/s)	K_D (M)	K_D (μM)
T1h	2.5	1.18E+04	5.54E-03	4.686E-07	0.468
Bmab-600	5.1	1.28E+04	4.20E-03	3.28E-07	0.328
Bmab-600	9.6	1.96E+04	6.00E-03	3.06E-07	0.306
Bmab-600	35.6	2.73E+04	5.34E-03	1.95E-07	0.195

[0148] 图 11 :通过加入锰 (Mn) 引起的非岩藻糖基化

[0149] 针对产生 T1h 单克隆抗体的 CHO-S 细胞系测试加入浓度高于培养基浓度 (0.005 μM) 的 Mn。试验以 80 万至 90 万个细胞 / 毫升的起始细胞数开始。在过程期间进行葡萄糖和氨基酸的常规进料,以满足细胞的营养需求。对样品进行定期取样以检验细胞生长、存活力及 IgG 滴度特征谱。在培养结束时收获培养基并且如本文别处描述地分析糖基化特征谱。

[0150] 以两组来完成试验。第一组是在振荡瓶中进行,而第二组是在 50L 生物反应器中进行。将锰加入培养基中并且在运行期间以指定间隔通过进料加入。

[0151] 图 11 显示将锰加入培养基和通过进料加入使非岩藻糖基化水平的增加。非岩藻糖基化特征谱对应于在 0.1mM、0.2mM 及 0.25mM 的情况下的第 10 天样品 ;以及在 0.075mM 及 0.23mM 的情况下的第 11 天样品。结果概述于以下表中 :

[0152] 表 6 :使用 0.1mM、0.2mM 和 0.25mM 锰 (Mn) 浓度的 10 天振荡瓶试验的聚糖特征谱

[0153]

所测试的 Mn 浓度	G0-GN	G0f-GN	G0	G0f	Man5	G1f-GN,G1	G1f	Man6,(G1f-GN)S1	G1FS1,具有 1G. 混合物 S1 的三触	G2f	G2FS1,具有小混合物	G2S2	其它混合物种类	非岩藻糖基化
0.1 mM	0.5	1.1	4.9	56.7	3.1	1.9	25.2	1.1	0.8	2.5	1.2	0.5	0.5	9.6
0.2 mM	0.6	0.9	6.3	55.1	4.2	2.3	24.1	1.2	0.8	2.4	1.1	0.5	0.7	12.3
0.25 mM	1.2	1	10.3	51.2	6.8	3.1	20.3	1.1	0.7	1.9	1	0.4	1	19.4

[0154] 表 7 :使用 0.075mM 和 0.23mM Mn 浓度 11 天 50L 批次运行的聚糖特征谱

[0155]

所检测的 Mn 浓度	G0-GN	G0f-GN	G0	G0f	Man5	G1f-GN,G1	G1f	Man6, (G1f-GN)S1	G1FS1, 具有 1G 混合物 S1 的三触角复合物	G2f	G2fS1, 具有小混合物	G2fS2	其它混合物种类	非岩藻糖基化
0.075mM	0.2	1	2	40.6	1.9	2.3	38.9	1.8	1.1	6.2	2.3	0.9	0.8	5.9
0.23 mM	0.6	1.5	4.5	42.4	7.3	3.7	29.1	2.1	1	4	2.3	0.3	1.1	14.5

[0156] 基于以上实验，随着锰的总浓度的增加观察到非岩藻糖基化百分比的增加。细胞生长、存活力和 IgG 滴度特征谱未受 Mn 添加的影响。

[0157] 图 12 :通过加入锰 (Mn) 增加 G0、Man5 和非岩藻糖基化水平。

[0158] 通过改变在培养基中的浓度来测试锰在 0.0025 μM 至 0.5mM 的范围中的效应。没有通过进料来加入锰。试验在振荡瓶中进行，并且在第 8 天针对糖基化特征谱来分析样品。可以观察到，随着锰浓度的增加，G0、Man5 和非岩藻糖基化水平也逐步增加。

[0159] 图 13 :铜浓度不具有对岩藻糖基化的效应。

[0160] 为了评估其它二价阳离子的效应，选择 Cu 用于研究，因为 Cu 也是在糖基化途径中的辅因子（针对唾液酸转移酶）。在振荡瓶中测试在培养基中 0.01 μM 至 200 μM 范围内的不同铜浓度。如图 3 所示，没有观察到以任何值的增加 / 效应 (G0、Man5 和非岩藻糖基化）。这证实铜离子不影响蛋白质中的非岩藻糖基化水平。

[0161] 图 14 :免疫球蛋白 G 的示意图。

[0162] 图 14 显示免疫球蛋白 G(IgG) 的示意图。IgG 包含两条相同的轻链（各自包含两个结构域，V_L和 V_H）和两条相同的重链（各自包含四个结构域，V_H、C_H1、C_H2 和 C_H3）。抗体结合表面由重链和轻链的可变结构域形成，并且效应子功能（例如补体活化以及结合细胞毒性细胞）由抗体的 Vc 区域介导。

[0163] 图 15 :N- 聚糖结构的命名法。

[0164] 图 15 显示不同 N- 聚糖的概述。一般地，术语“N- 糖基化”意指氨基酸残基天冬酰胺 (N) 的糖基化。这里，寡糖链由寡糖基转移酶附接至存在于三肽序列 Asn-X-Ser 或 Asn-X-Thr 中的那些天冬酰胺残基，其中 X 可以是除了 Pro 之外的任何氨基酸。

[0165] 本文显示的实验清楚地证实 :

[0166] a) 糖蛋白的岩藻糖含量可以通过在蛋白质表达过程中改变培养基和进料中的锰或锰离子的总浓度而进行操纵。

[0167] b) 增加锰或锰离子的总浓度导致糖蛋白的非岩藻糖基化增加或导致糖蛋白的糖基化模式中的岩藻糖含量降低。

[0168] c) 在免疫配体（如具有 Fc 区域的抗体）中，在存在升高的锰或锰离子浓度的情况下的蛋白质表达导致了（i）较高的非岩藻糖基化程度以及（ii）增加的 ADCC。

[0169] d) 在这些免疫配体中，增加非岩藻糖基化程度不导致增加的 CDC。

[0170] e) 相反地，免疫配体（如具有 Fc 区域的抗体）的去糖基化不导致增加的 ADCC。

[0171] f) 除了非岩藻糖基化，免疫配体（如具有 Fc 区域的抗体）的去糖基化可以导致这样的免疫配体的功能活性的丧失，特别是如果这样的功能活性与如效应子功能和 / 或 ADCC 的活性有关的话。

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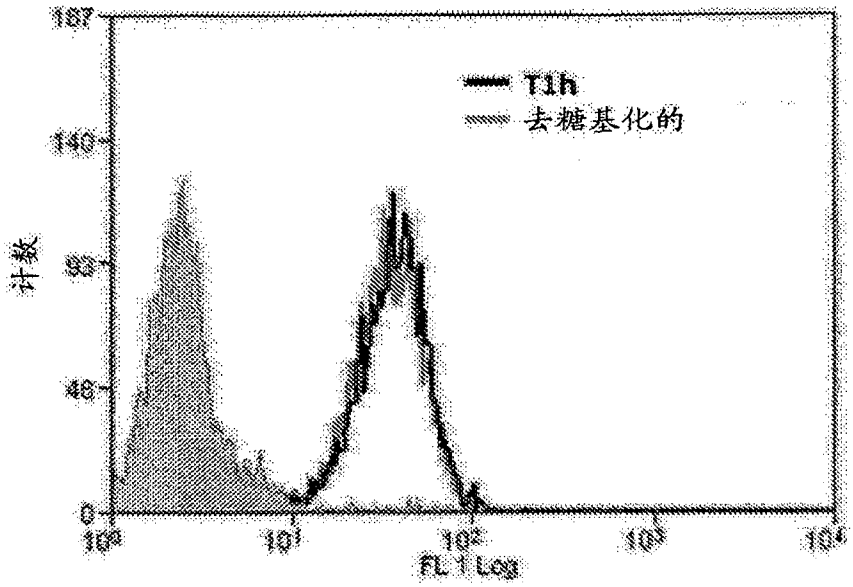

T1h
去糖基化的
计数
FL 1 Log

图 1

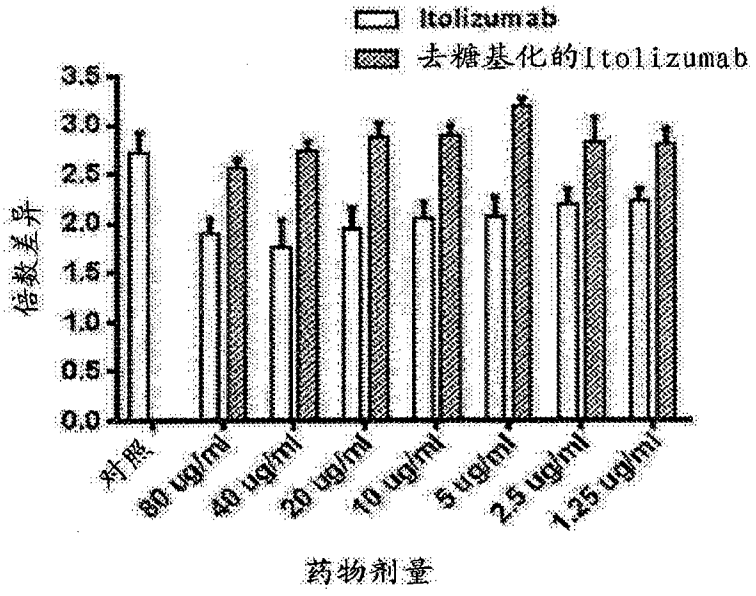

Itolizumab
去糖基化的Itolizumab
倍数差异
对照
80 ug/ml
40 ug/ml
20 ug/ml
10 ug/ml
5 ug/ml
2.5 ug/ml
1.25 ug/ml
药物剂量

图 2

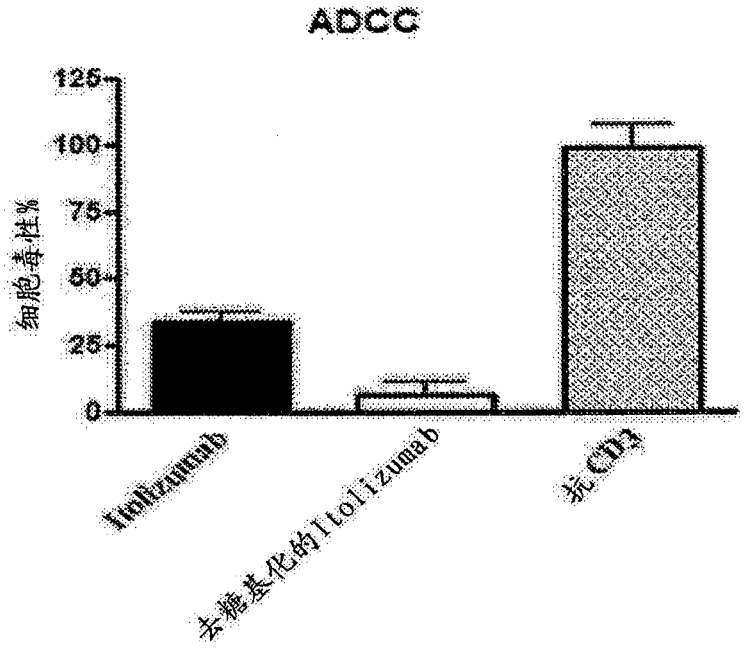

ADCC
细胞毒性%
125
100
75
50
25
0
Tuhrxetuub
去糖基化的Tolizumab
抗CD3

图 3

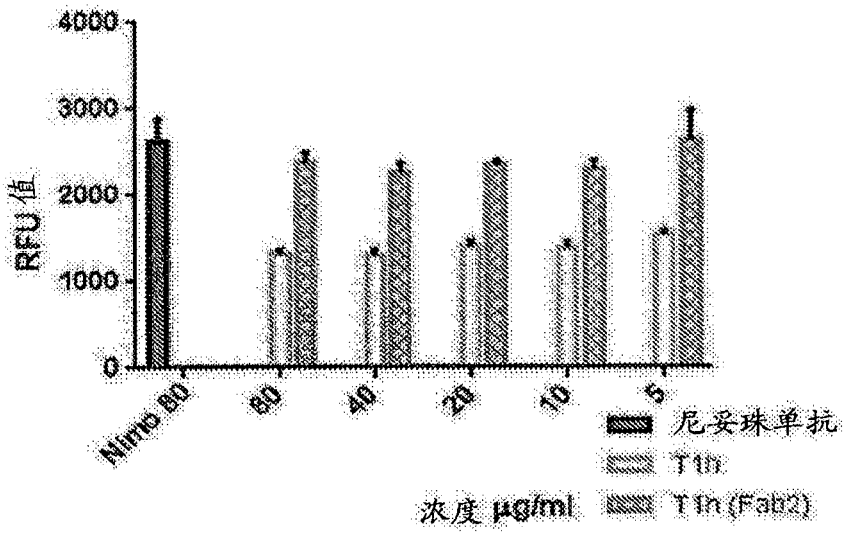

RFU值
4000
3000
2000
1000
0
Nimo 80
80
40
20
10
5
浓度 μg/ml
尼妥珠单抗
T1h
T1h(Fab2)

图 4

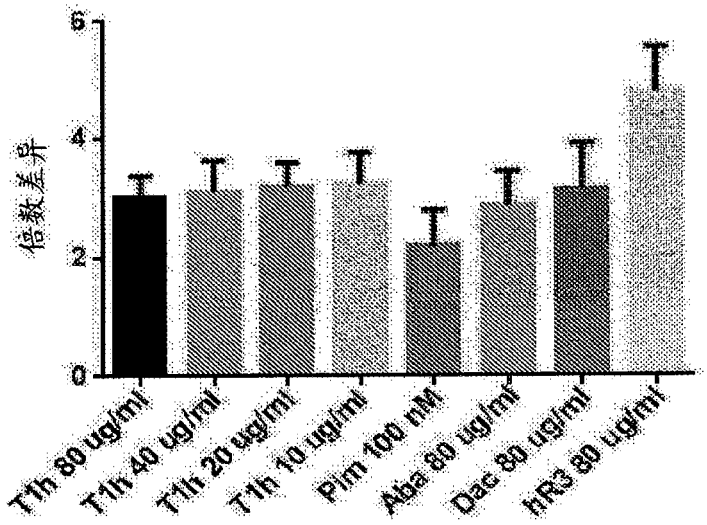

倍数差异
6
4
2
0
T1h 80 ug/ml
T1h 40 ug/ml
T1h 20 ug/ml
T1h 10 ug/ml
Pim 100 nM
Aba 80 ug/ml
Dac 80 ug/ml
hR3 80 ug/ml

图 5a

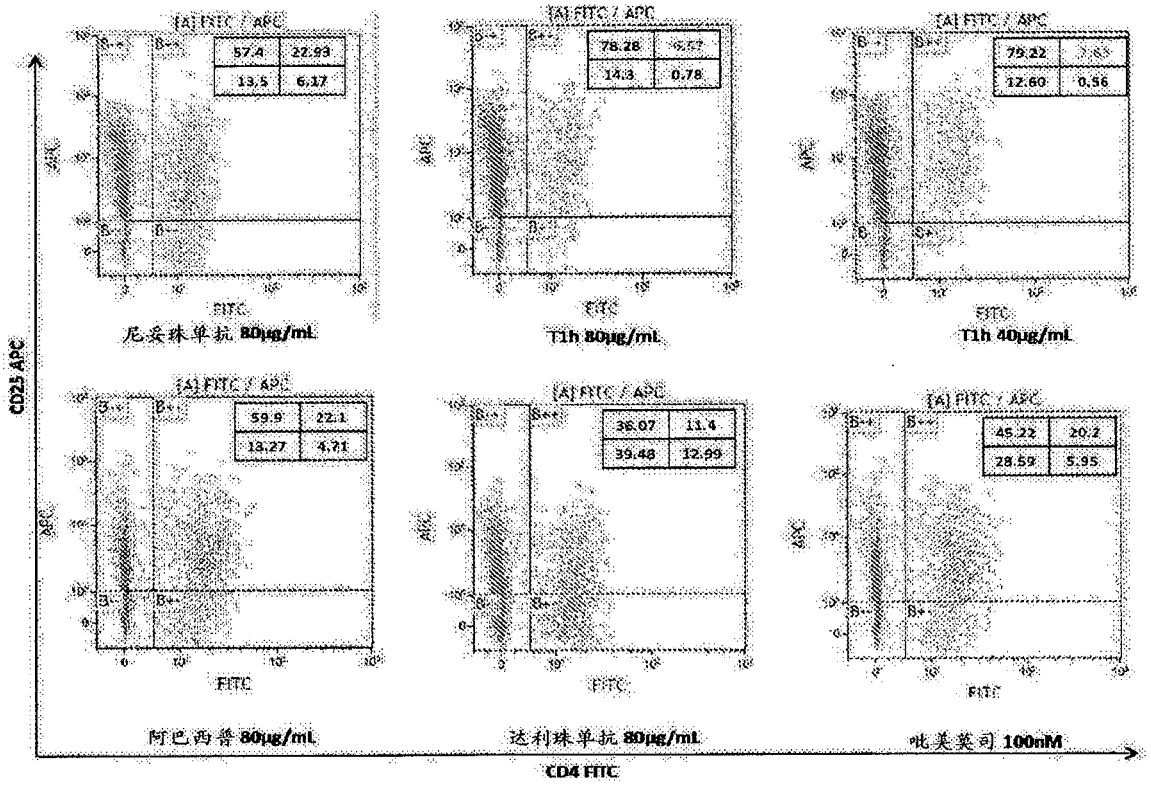

[A] FITC / APC
57.4
22.93
13.5
6.17
尼妥珠单抗 80μg/mL
78.26
14.3
0.78
T1h 80μg/mL
79.22
12.60
0.56
T1h 40μg/mL
59.9
22.1
13.27
4.71
阿巴西普 80μg/mL
36.07
11.4
39.48
12.99
达利珠单抗 80μg/mL
45.22
20.2
28.59
5.95
吡美莫司 100nM
CD25 APC
CD4 FITC

图 5b

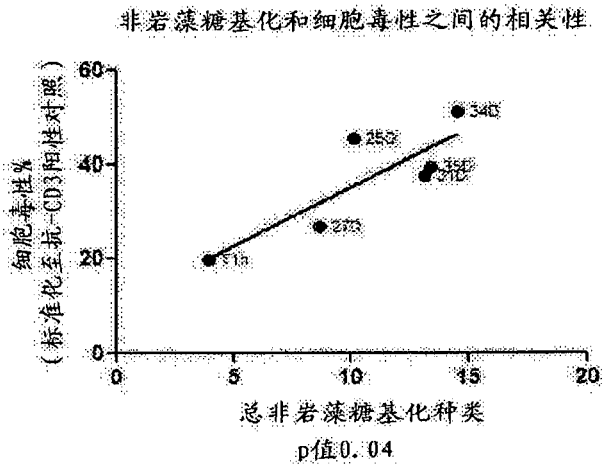

非岩藻糖基化和细胞毒性之间的相关性
细胞毒性%
（标准化至抗-CD3阳性对照）
60
40
20
0
0
5
10
15
20
总非岩藻糖基化种类
p值0.04

图 6

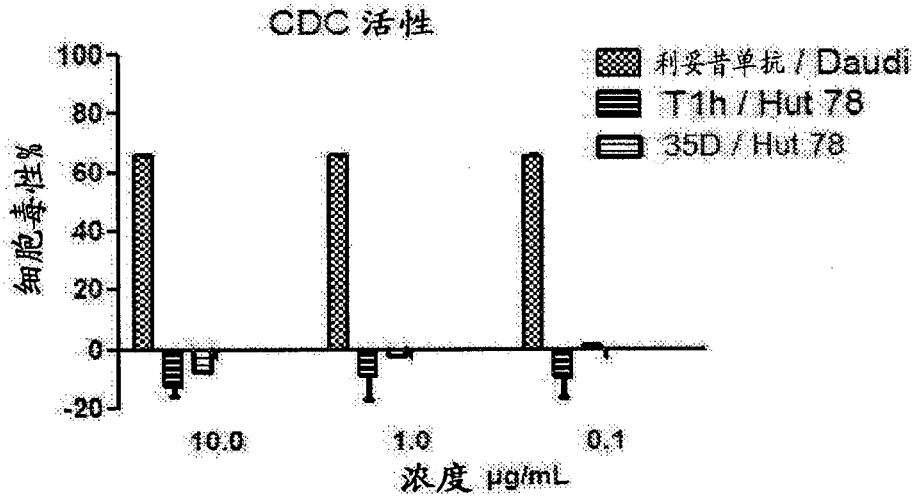

CDC 活性
细胞毒性%
100
80
60
40
20
0
-20
10.0
1.0
0.1
浓度 μg/mL
利妥昔单抗 / Daudi
T1h / Hut 78
35D / Hut 78

图 7

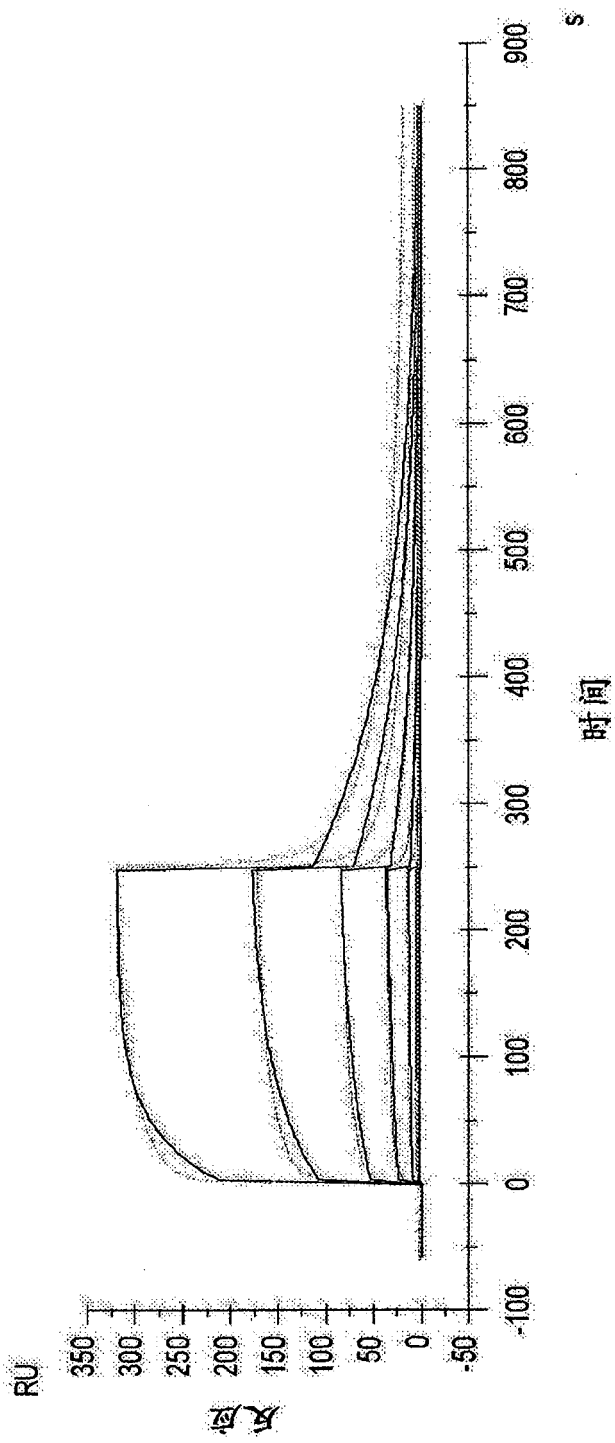

RU
350
300
250
200
150
100
50
0
-50
反应
-100
0
100
200
300
400
500
600
700
800
900
s
时间

图 8

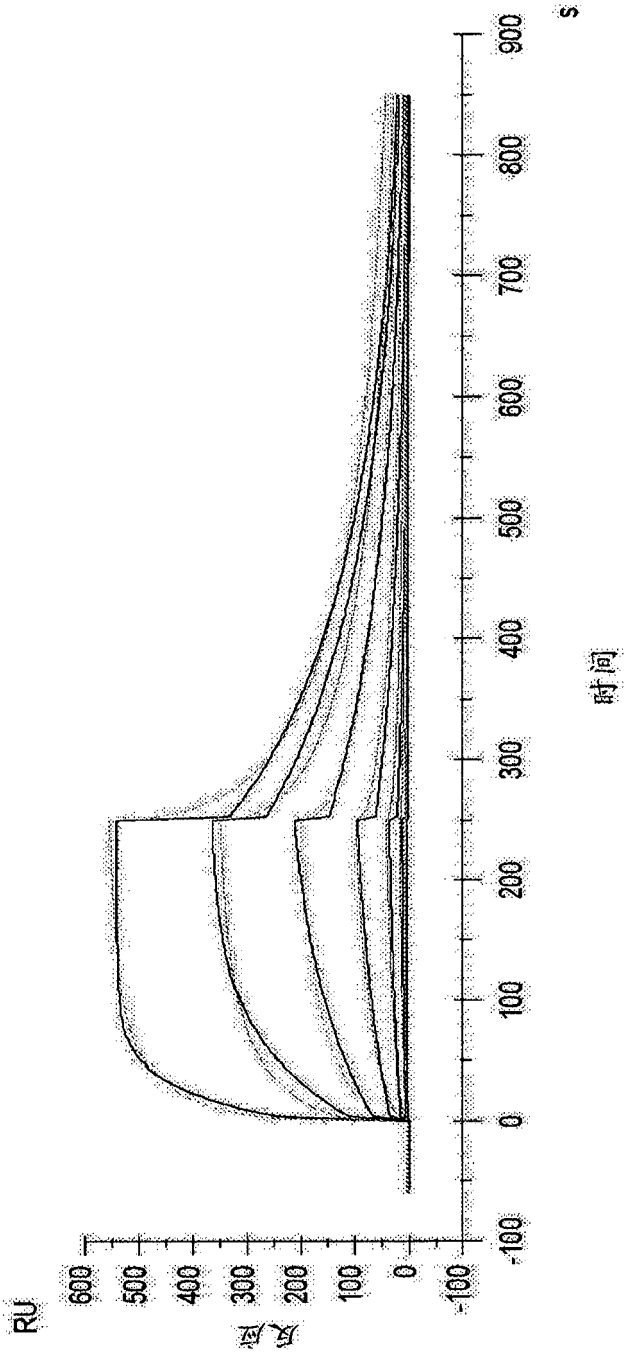

RU
600
500
400
300
200
100
0
-100
反应
-100
0
100
200
300
400
500
600
700
800
900
s
时间

图 9

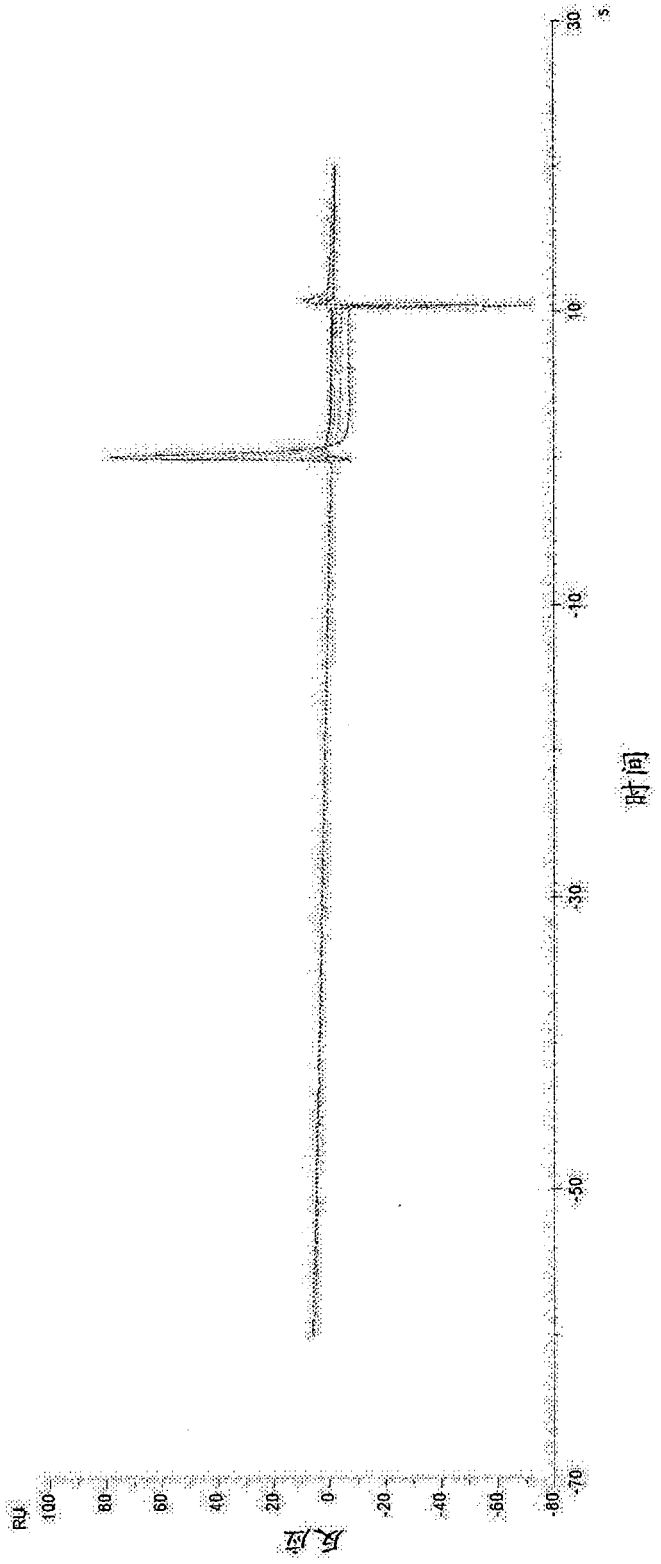


图 10

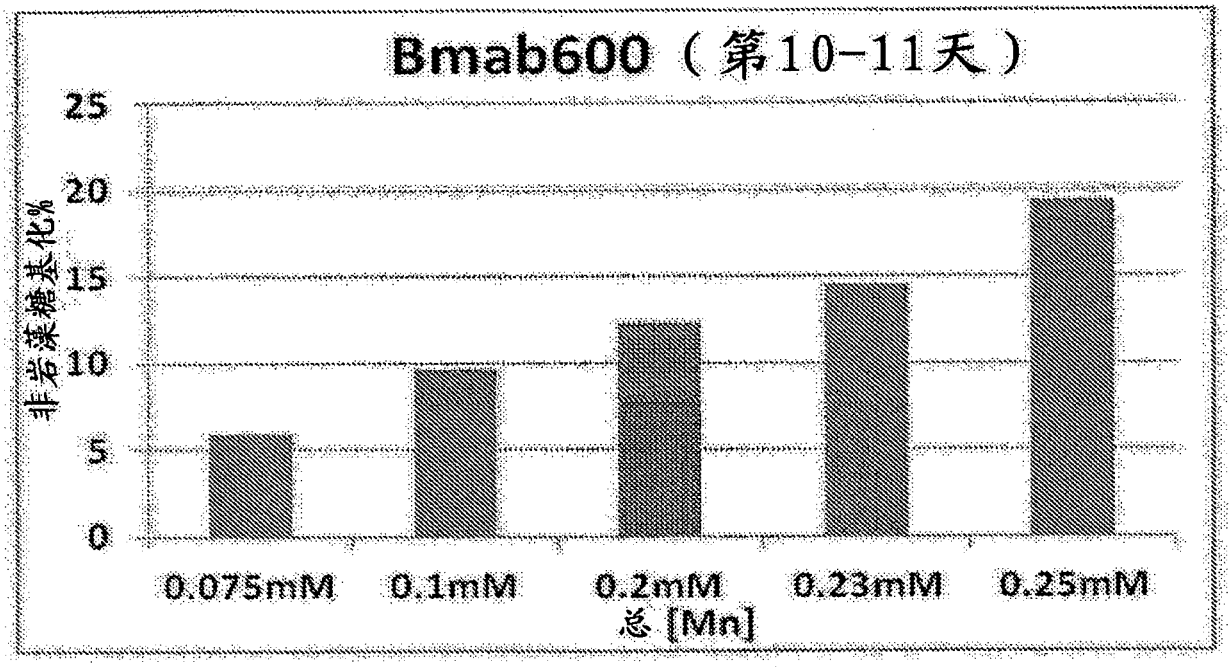

Bmab600（第10-11天）
非岩藻糖基化%
25
20
15
10
5
0
0.075mM
0.1mM
0.2mM
0.23mM
0.25mM
总 [Mn]

图 11

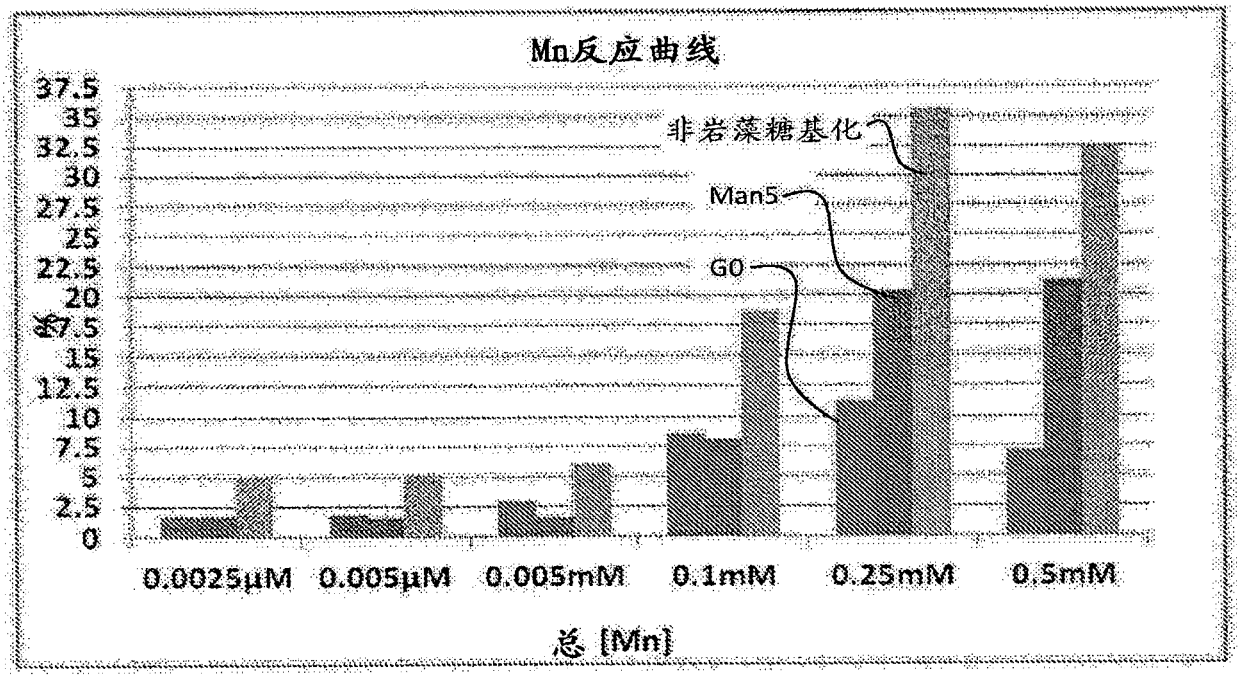

Mn反应曲线
37.5
35
32.5
30
27.5
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
非岩藻糖基化
Man5
G0
0.0025μM
0.005μM
0.005mM
0.1mM
0.25mM
0.5mM
总 [Mn]

图 12

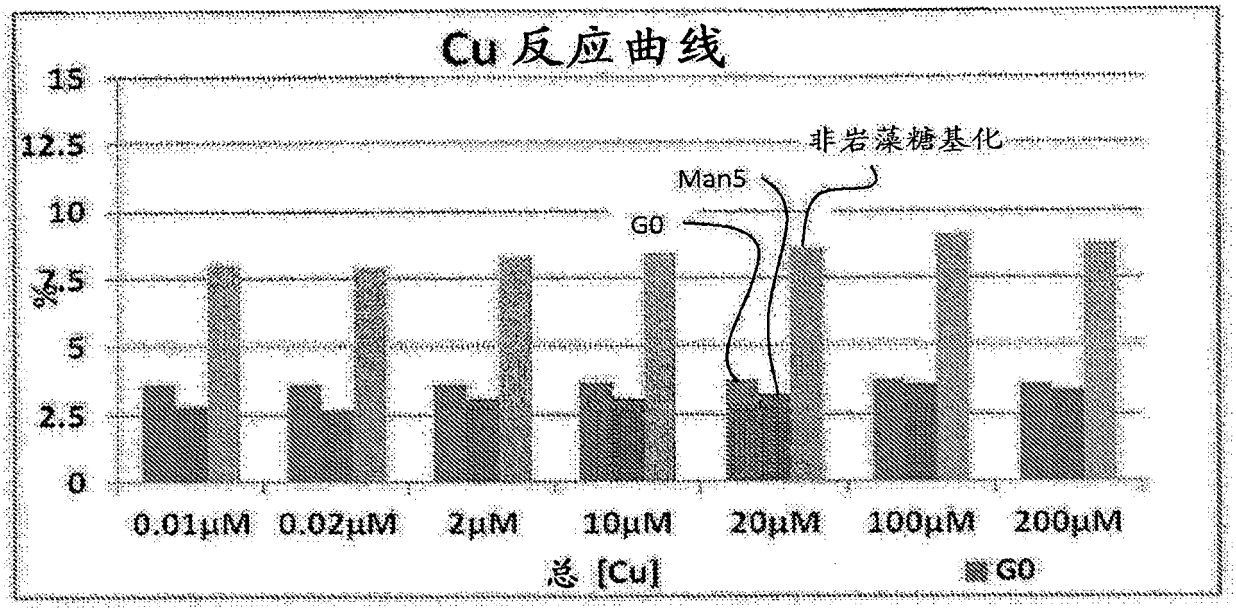

Cu反应曲线
15
12.5
10
7.5
5
2.5
0
%
非岩藻糖基化
Man5
G0
0.01μM
0.02μM
2μM
10μM
20μM
100μM
200μM
总 [Cu]
G0

图 13

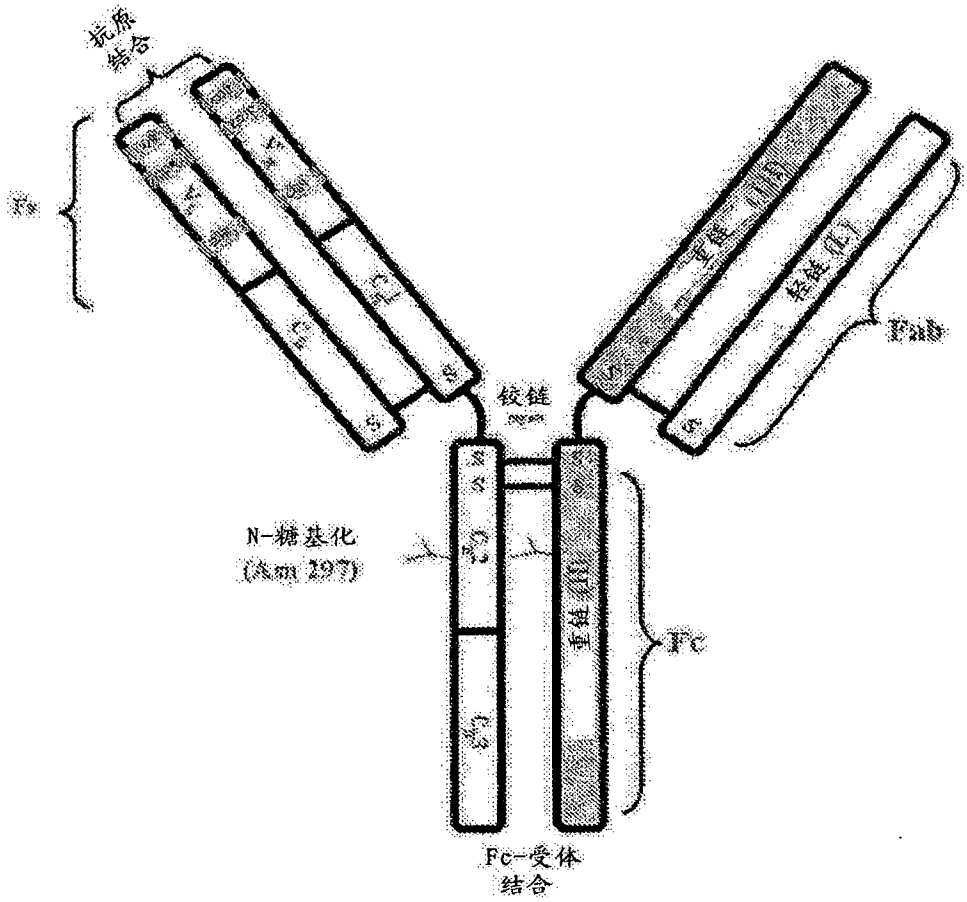
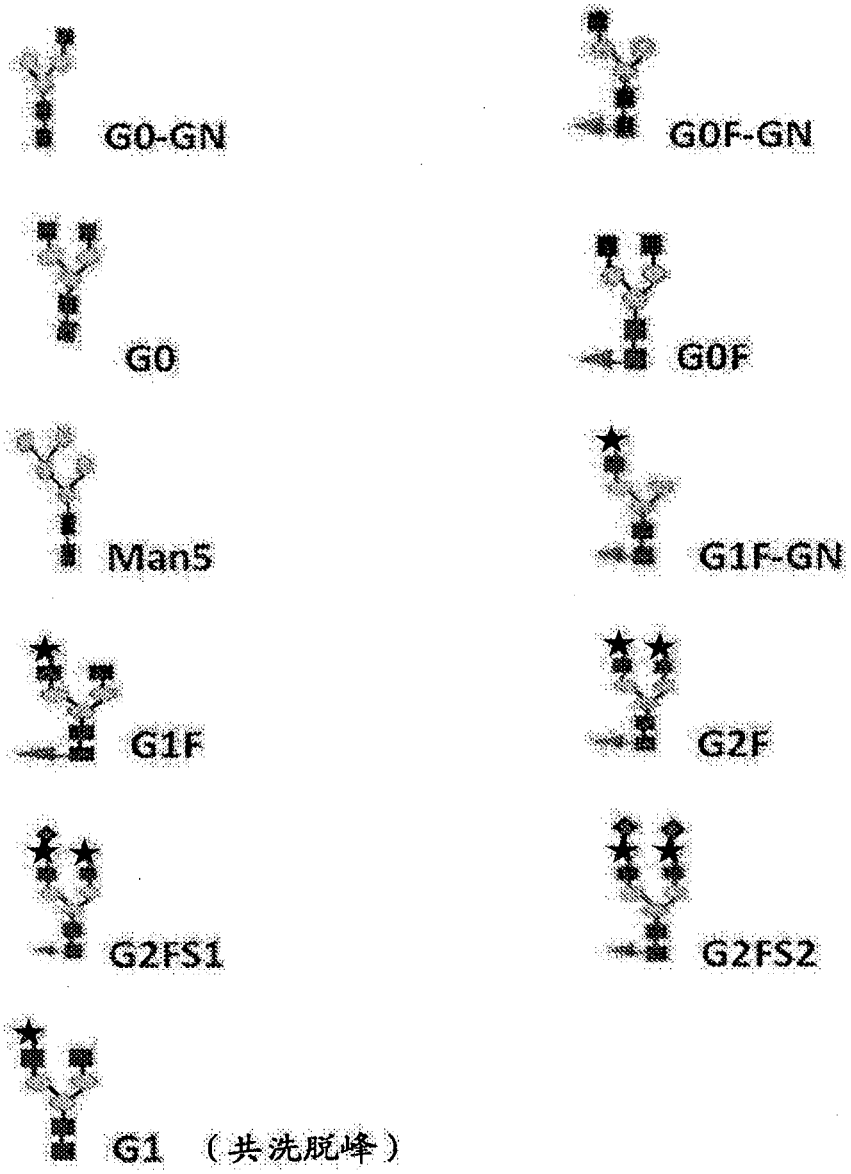


图 14



方块-N-乙酰基葡糖胺，圆圈-甘露糖，
星形-半乳糖，三角形-岩藻糖，菱形-唾液酸

图 15