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WO-A1-2013/120939
WO-A1-2014/179184
PEGON JULIE N ET AL: "Factor VIII and von Willebrand factor are ligands for the carbohydrate-receptor Siglec-5", HAEMATOLOGICA-THE HEMATOLOGY JOURNAL, vol. 97, no. 12, December 2012 (2012-12-01), pages 1855 - 1863, XP002763767
LAI JESSE DEREK ET AL: "To clear or to fear: An innate perspective on factor VIII immunity", CELLULAR IMMUNOLOGY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 301, 28 October 2015 (2015-10-28), pages 82 - 89, XP029420136, ISSN: 0008-8749, DOI: 10.1016/J.CELLIMM.2015.10.011
SOLECKA B A ET AL: "Site-specific analysis of von Willebrand factor O-glycosylation.", JOURNAL OF THROMBOSIS AND HAEMOSTASIS : JTH APR 2016, vol. 14, no. 4, April 2016 (2016-04-01), pages 733 - 746, XP002763768, ISSN: 1538-7836
K. CANIS ET AL: "The plasma von Willebrand factor O -glycome comprises a surprising variety of structures including ABH antigens and disialosyl motifs", JOURNAL OF THROMBOSIS AND HAEMOSTASIS, vol. 8, no. 1, 1 January 2010 (2010-01-01), pages 137 - 145, XP055202964, ISSN: 1538-7933, DOI: 10.1111/j.1538-

Fortsættes ...

7836.2009.03665.x

LENTING P J ET AL: "von Willebrand factor: the old, the new and the unknown.", JOURNAL OF THROMBOSIS AND HAEMOSTASIS : JTH DEC 2012, vol. 10, no. 12, December 2012 (2012-12-01), pages 2428 - 2437, XP002763769, ISSN: 1538-7836

DESCRIPTION

Description

FIELD OF THE INVENTION

[0001] The present invention relates to glycosylated polypeptides based on a mammalian protein exhibiting reduced immune response or increased immune tolerance due to modulated SIGLEC binding and to methods of treatment using the glycosylated polypeptide. The invention further relates to protein complexes exhibiting reduced immune response or increased immune tolerance due to modulated SIGLEC binding and to methods of treatment using the protein complexes.

BACKGROUND OF THE INVENTION

[0002] Hemophilia is a group of hereditary genetic disorders that impair the body's ability to control blood clotting or coagulation. In its most common form, Hemophilia A, clotting factor VIII (FVIII) is deficient, Hemophilia A occurs in about 1 in 5,000-10,000 male births. The FVIII protein is an essential cofactor in blood coagulation with multifunctional properties. The deficiency of FVIII can be treated with plasma-derived concentrates of FVIII or with recombinantly produced FVIII. The treatment with FVIII concentrates has led to a normalized life of the hemophilia patients. Historically, Hemophilia A has been treated with FVIII originating from human blood plasma. In blood plasma, under normal conditions, the FVIII molecule is always associated with its cofactor; von Willebrand factor (vWF), which stabilizes the FVIII molecule from different forms of degeneration.

[0003] Many processes have been described for purification of Factor VIII from plasma or cultures which recombinantly produce Factor VIII (rFVIII) with or without the presence of von Willebrand Factor. In the 90 's, the first recombinant FVIII (rFVIII) products were marketed, divided in full-length rFVIII molecules, mimicking the main form of FVIII in blood plasma, and B-domain deleted rFVIII molecules (Eriksson et al., 2001), in which one inactive portion (the B-domain) has been removed, both with a high degree of purity (all without vWF).

[0004] Hemophilia A patients are treated with FVIII on demand or as a prophylactic therapy administered several times a week. For prophylactic treatment 15-25 IU/kg bodyweight of FVIII is administered three times a week which is necessary due to the constant need of FVIII and its short half-life in the blood system, which is in humans only about 11 hours. (Ewenstein et al., 2004).

[0005] In frequent cases the constant treatment with exogenously administered FVIII causes a response of the patient's immune system (Saenko et al., *Haemophilia* 8:1-11 (2002)), which presents a serious limitation to the therapy.

[0006] Presently, the most common option to achieve immune tolerance in patients with haemophilia A (congenital FVIII-deficiency) and inhibitors is immune tolerance induction (ITI), where high doses of FVIII are administered for prolonged periods of time. However, the treatment can take up to two years, remains unsuccessful in approximately 30% of patients, is extraordinarily costly, and cannot be used in a prophylactic manner to suppress the initial development of inhibitory antibodies.

[0007] Thus, approaches to attenuate the immune response are needed. One promising approach is the optimization of the glycosylation of either FVIII or its binding partner vWF.

[0008] For example, WO 2014/176125 A1 relates to immune conjugates for inducing antigen specific immune tolerance to FVIII. The immune conjugates are FVIII proteins conjugated to specific glycan ligands that target SIGLECs expressed on B-cells, namely SIG-1 or SIG-10 (or the ortholog SIG-G). The glycans ligands are in particular coupled to liposomes into which FVIII is introduced.

[0009] Sialic Acid Binding Immunoglobulin Lectins (SIGLECs) comprise a family of 15 human and 9 murine cell surface receptors that are expressed on various white blood cells of the immune system with the exception of most T-cells in mouse and man. The SIGLECs are located on different cell types and bind to different glycans structures (reviewed in Paulson et al., 2012). For example, a binding of vWF and FVIII to SIG-5 has been demonstrated (Pegon 2012). However, the mechanism of binding remains unknown.

[0010] A different approach is described in WO 2014/179184 A1. The authors suggest reducing undesired antibody immune responses and inducing immune tolerance of blood coagulation factors, such as FVIII by addition of SIGLEC ligands. The SIGLEC ligands are selected from 9-N-biphenylcarboxyl-NeuAca2-6Gal~I-4GlcNAc (6'-BPCNeuAc), NeuAca2-6GalwI-4GlcNAc and NeuAca2-6GalwI-4(6-sulfo)GlcNAc. The SIGLEC ligand is linked to the coagulation factor via a water-soluble polymer.

[0011] Lai et al. (2015) is a review article examining three distinct factors contributing to the fate of FVIII almost immediately after infusion, namely the characteristics of the protein, the cell and the microenvironment

[0012] Solecka et al. (2016) describes a site-specific analysis of the O-glycosylation of vWF.

[0013] Canis et al. (2009) is a further study investigating the O-glycosylation sites in human plasma-derived vWF.

[0014] Lenting et al. (2012) is a review article about the structure and function of vWF. The

article discusses the unique attributes of vWF with respect to its multimeric structure and the unusual mechanisms regulating its participation in the hemostatic process.

[0015] WO 2013/120939 A1 relates to a modified vWF polypeptide with an increased binding affinity to FVIII as well as a complex comprising FVIII and the modified vWF polypeptide.

SUMMARY OF THE INVENTION

[0016] The present invention is, *inter alia*, based on the finding that the glycan structure naturally occurring in plasma derived proteins, in particular vWF enables an interaction with a group of SIGLECs, in particular SIG-5, SIG-7, SIG-8 and SIG-9. Moreover, the inventors found that by modification of the glycan structure on a protein the interaction with SIGLECs, such as SIG-5, SIG-7, SIG-8 and SIG-9 can be increased. This increase leads to a reduced immune response and/or increased immune tolerance of a patient to which the protein is administered.

[0017] Thus, according to a first aspect, the invention provides a glycosylated polypeptide comprising an amino acid sequence being at least 90 % identical to at least a fragment of human vWF (SEQ ID NO: 1), wherein said glycosylated polypeptide contains one or more clusters of O-glycosylation sites, wherein said clusters contain at least two O-glycosylation sites and one or more sialylated O-glycans, wherein the combined number of sialylated core 2 and extended core 1 O-glycans of the glycosylated polypeptide is higher than the number of sialylated core 2 and extended core 1 O-glycans of the human vWF or the fragment thereof and wherein the glycosylated polypeptide shows an increased binding affinity to one or more SIGLECs, selected from the group consisting of SIG-5, SIG-7, SIG-8, and SIG-9 compared to the human vWF or fragment thereof.

[0018] The inventors have specifically defined a glycan structure that is on the one hand needed for interaction with the SIGLECs but also the addition of which leads to an increased binding to the SIGLECs. Responsible for this are sialylated core 2 O-glycans and/or extended core 1 O-glycans.

[0019] A protein with a glycan composition including sialylated core 2 O-glycans and/or sialylated extended core 1 O-glycans, in particular sialylated core 2 O-glycans, can be used to modify the immune response of a patient to a therapeutic protein by combined administration.

[0020] Thus, according to a second aspect the invention relates to the use of a human VWF containing one or more sialylated O-glycans and exhibiting increased binding to one or more SIGLECs, selected from SIG-5, SIG-7, SIG-8, and SIG-9 for reducing the immune response of a second protein or increasing the immune tolerance of a patient to a second protein, wherein the second protein is in particular a therapeutic protein.

[0021] Using the modification of the glycosylated polypeptide with a binding affinity to SIGLECs it is not only possible to directly modify the SIGLEC binding affinity of the modified polypeptide

itself, but also of a protein complex or composition, which the glycosylated polypeptide is a part of, such as the complex of factor VIII (FVIII) and von-Willebrand-factor (vWF).

[0022] Thus, according to a third aspect the invention provides a protein composition comprising a first and a second polypeptide, wherein the first polypeptide is a glycosylated polypeptide according to the first aspect and the second polypeptide contains an amino acid sequence homologous or identical to a second mammalian, in particular human protein, wherein - compared to the second polypeptide - the composition has an increased binding affinity to one or more SIGLECs selected from SIG-5, SIG-7, SIG-8, and SIG-9. The first and second polypeptide of the composition according to the third aspect preferably form a protein complex.

[0023] According to a fourth aspect, the invention provides an isolated polynucleotide that comprises a nucleic acid sequence encoding a glycosylated polypeptide according to the first aspect of the invention. In a fifth aspect the invention also relates to expression vector comprising a polynucleotide according to the fourth aspect of the invention.

[0024] The glycosylated polypeptide according to the first aspect, in particular vWF or FVIII and the composition according to the third aspect, in particular a complex of FVIII and vWF, are - due to the reduced immune response - particularly useful in medical treatment.

[0025] Thus, according to a fourth aspect, the invention provides a glycosylated polypeptide defined according to the first aspect or a composition defined according to the third aspect for use in the treatment or prevention of a bleeding disorder.

FIGURES

[0026]

Fig. 1

shows the results of a binding test of vWF to different SIGLECs. The absorbance at 492 nm is proportional to the vWF bound to the respectively identified SIGLEC or the controls. SIG-2, SIG-5, SIG-7, SIG-F, SIG-9 and SIG-10 were immobilized via protein A on a microtiter plate at 500 ng/well. Biotinylated vWF was added at a concentration of 0 to 0.8 µg/mL and after washing, binding was visualized with HRP-conjugated streptavidin and absorbance measured at 492 nm. Anti-vWF and Anti-Chicken IgY was used for control.

Fig. 2

shows a schematic representation of the vWF domain structure including N- and O-glycosylation, V8 protease cleavage sites and fragments resulting after V8 protease cleavage.

Fig. 3

shows the results of a binding test of an N-terminal and a C-terminal fragment of vWF to

SIGLECs SIG-5, SIG-7, SIG-F and SIG-9. The absorbance at 492 nm is proportional to the vWF fragment bound to the respectively identified SIGLEC or the control. SIG-5, SIG-7, SIG-F and SIG-9 were immobilized via protein A on a microtiter plate at 500 ng/well. Biotinylated N-terminal VWF fragment (dark grey bars) and C-terminal vWF fragment (light grey bars) were added at a concentration of 1 µg/mL and after washing, binding was visualized with HRP-conjugated streptavidin and absorbance measured at 492 nm. Anti-Chicken IgY was used as negative control.

Fig. 4

shows the results of a binding test of the vWF N-terminal fragment, in desialylated, de-N-glycosylated and untreated form. The absorbance at 492 nm is proportional to the vWF N-terminal fragment bound to the respectively identified SIGLEC or the control. The N-terminal VWF fragment prior to digestion is represented by white bars, the PNGase F de-N-glycosylated fragment by grey bars and the desialylated fragment by black bars. SIG-5, SIG-7, SIG-F and SIG-9 were immobilized via protein A on a microtiter plate at 500 ng/well. The biotinylated N-terminal vWF fragments (prior to digestion, digested with PNGase For Sialidase A) were added at a concentration of 8 µg/mL and after washing, binding was visualized with HRP-conjugated streptavidin and absorbance was measured at 492 nm. Anti-Chicken IgY was used for control.

Fig. 5

shows the results of a binding test of the O-glycosylation Cluster I and Cluster II to SIGLECs. The absorbance at 492 nm is proportional to the Cluster I fragment (light grey bars) or Cluster II fragment (dark grey bars) bound to the respectively identified SIGLEC or the control. SIG-5, SIG-7, SIG-F, SIG-9 and SIG-10 were immobilized via protein A on a microtiter plate at 500 ng/well. Biotinylated Cluster I and Cluster II were added at a concentration of 4 µg/mL and after washing, binding was visualized with HRP-conjugated streptavidin and absorbance measured at 492 nm. Anti-Chicken IgY was used as a negative control.

Fig. 6

shows the results of a binding test of the O-glycosylation Cluster II to SIGLECs before and after treatment with Sialidase A. The absorbance at 492 nm is proportional to the untreated Cluster II fragment (light grey bars) or the Cluster II fragment digested with Sialidase A (dark grey bars) bound to the respectively identified SIGLEC or the control. SIG-5, SIG-7, SIG-F and SIG-9 were immobilized via protein A on a microtiter plate at 500 ng/well. Biotinylated Cluster II prior to digestion (light grey bars) and digested with Sialidase A was added at a concentration of 2 µg/mL and after washing, binding was visualized with HRP-conjugated streptavidin and absorbance measured at 492 nm. Anti-Chicken IgY was used as a negative control.

Fig. 7

shows a schematic representation of recombinantly expressed vWF fragments Seq11 and Seq12.

Fig. 8

shows MALDI MS spectra of the O-glycopeptide isolated from Seq11 after tryptic/chymotryptic digestion; Sialidase A digestion and lectin enrichment. The identified peptide sequence is

KVTLNPSDPEHCQICHCDVNLTCACQEPGGLVWPPTDAPVSPTTLY

VEDISEPPLHGSAW (SEQ ID NO: 6), the last four amino acids (underlined) correspond to the tag attached to the C-terminus of the sequence. Upper spectrum shows the fully O-glycosylated glycopeptide, bottom spectrum shows the same glycopeptide after O-glycosidase digestion.

Fig. 9

shows MALDI MS spectra of the O-glycopeptide isolated from Seq12 after tryptic/chymotryptic digestion; sialidase A digestion and lectin enrichment. The identified peptide sequence is

KVTLNPSDPEHCQICHCDVNLTCACQEPGGLVWPPTDAPVSPTTLY

VEDISEPPLHQEPGGL VWPPTDAPVSPTTL YVEDISEPPLHQEPGGL VWPPTDAPVSPTTLYVEDISEPPLHGSAW (SEQ ID NO: 7), the last four amino acids (underlined) correspond to the tag attached to the C-terminus of the sequence. The upper spectrum shows the fully O-glycosylated glycopeptide, bottom spectrum shows the same glycopeptide after O-glycosidase digestion.

Fig. 10

shows the results of a binding test of recombinant polypeptides Seq11 and Seq12 to SIGLECs. The absorbance at 492 nm is proportional to Seq11 (dark grey bars) or Seq12 (light grey bars) bound to the respectively identified SIGLEC or the control. SIG-5, SIG-7, SIG-F and SIG-9 were immobilized via protein A on a microtiter plate at 500 ng/well. Strep-Tag bearing sequences were applied on the plate at equal molar concentrations of 42 nM and after washing, binding was visualized with HRP-conjugated Streptactin and absorbance measured at 492 nm. Anti-Chicken IgY was used as a negative control and anti vWF pAb as a positive control.

Fig. 11

shows the results of a binding test of recombinant polypeptides Seq11 and Seq12 after Sialidase A treatment to SIGLECs. The absorbance at 492 nm is proportional to Seq11 (dark grey bars) or Seq12 (light grey bars) bound to the respectively identified SIGLEC or the control. SIG-5, SIG-7, SIG-F and SIG-9 were immobilized via protein A on a microtiter plate at 500 ng/well. Strep-Tag bearing sequences were enzymatically desialylated and applied on the plate at equal molar concentrations of 42 nM and after washing, binding was visualized with HRP-conjugated Streptactin and absorbance measured at 492 nm. Anti-Chicken IgY was used as a negative control and anti-vWF pAb as a positive control.

Fig. 12

shows the results of a concentration dependent binding of recombinant polypeptide Seq11 to SIGLECs and Scatchard analysis of the specific binding curves.

Fig. 13

shows the results of a concentration dependent binding of recombinant polypeptide Seq12 to SIGLECs and Scatchard analysis of the specific binding curves.

Fig. 14

shows the summary of the K_D values obtained from the Scatchard analysis performed for curves presented in Fig. 12 and Fig. 13.

Fig. 15

shows the dissociation affinity constants (KD) values calculated for the binding of Seq11, Seq12 and fulllength plasmatic VWF to recombinant FVIII. Data was obtained by SPR.

Fig. 16

Effect of N- and C-terminal VWF fragments on IL-12p70 and IFN- γ . moDCs were cultivated with various concentrations of the VWF-fragments either without (left column) or with (right column) the addition of 0.1 μ g/ml LPS. Extracellular levels of the cytokines were determined simultaneously via a cytometric bead array. IL-12p70 and IFN- γ levels of the unstimulated cells were for most donors below the detection limit (bd, 0.6 pg/ml for IL-12p70 and 1.8 pg/ml for IFN- γ). Data are presented as mean $\pm$ SEM with each dot representing one donor.

Fig. 17

shows the results of phosphorylation of SIGLECs and adaptor molecules SHP-1 and SHP-2 involved in SIGLEC-signaling, following stimulation of moDC for 10 minutes with 500 nM of the N-terminal fragment of VWF. Cells stimulated with the same volume of 100 mM NaCl served as a control. Analysis of immunoreceptor-phosphorylation in the cell lysates was carried out with the Proteome Profiler Human Phospho-Immunoreceptor Array Kit. Results are shown as mean pixel density $\pm$ SEM of 2-4 individual experiments.

DETAILED DESCRIPTION OF THE INVENTION

[0027] In order to provide a clear and consistent understanding of the specification and claims, and the scope to be given such terms, the following definitions are provided.

Definitions

[0028] A "peptide" as used herein may be composed of any number of amino acids of any type, preferably naturally occurring amino acids, which, preferably, are linked by peptide bonds. In particular, a peptide comprises at least 3 amino acids, preferably at least 5, at least 7, at least 9, at least 12, or at least 15 amino acids. Furthermore, there is no upper limit for the length of a peptide. However, preferably, a peptide according to the invention does not exceed a length of 500 amino acids, more preferably it does not exceed a length of 300 amino acids; even more preferably it is not longer than 250 amino acids.

[0029] Thus, the term "peptide" includes "oligopeptides", which usually refer to peptides with a length of 2 to 10 amino acids, and "polypeptides" which usually refer to peptides with a length of more than 10 amino acids.

[0030] The term "protein" as used herein refers to a peptide with at least 60, at least 80, preferably at least 100 amino acids. The terms "polypeptide" and "protein" are used interchangeably. The polypeptides and proteins as used herein include chemically synthesised

proteins as well as naturally synthesised proteins which are encoded by genes. The polypeptides or proteins may be obtained from a natural source, such as human blood or produced in cell culture as recombinant proteins.

[0031] As used herein the term "mammalian protein" relates to the naturally occurring mammalian protein, i.e. a protein naturally expressed by a mammalian organism.

[0032] Therefore, the mammalian protein has a naturally occurring amino acid sequence and naturally occurring post-translational modifications, such as glycosylation. According to the invention, the terms mammalian protein and naturally occurring mammalian protein may be used interchangeably.

[0033] As used herein the term "human protein" relates to the naturally occurring human protein, i.e. a protein naturally expressed by a human organism. Therefore, the human protein has a naturally occurring amino acid sequence and naturally occurring post-translational modifications, such as glycosylation. According to the invention, the terms human protein and naturally occurring human protein are used interchangeably.

[0034] "Recombinant proteins" or "recombinant polypeptides" as used herein are those which are encoded by transgenes introduced into the cells by molecular biology techniques. Proteins can be modified by chemical methods or by enzymes in post-translational processes.

[0035] The term "fusion protein" according to the invention relates to proteins created through the joining of two or more genes, cDNAs or sequences that originally coded for separate proteins/peptides. The genes may be naturally occurring in the same organism or different organisms or may be synthetic polynucleotides.

[0036] The term "therapeutic protein" as used herein relates to proteins or polypeptides with a therapeutic effect, i.e. proteins used as active pharmaceutical ingredient.

[0037] The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity". For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the no brief option) is used as the percent identity and is calculated as follows:

[0038] The transitional term "comprising", which is synonymous with "including," "containing," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. The transitional phrase "consisting of" excludes any element, step,

or ingredient not specified in the claim, except for impurities ordinarily associated therewith. When the phrase "consists of" appears in a clause of the body of a claim, rather than immediately following the preamble, it limits only the element set forth in that clause; other elements are not excluded from the claim as a whole. The transitional phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. "A 'consisting essentially of' claim occupies a middle ground between closed claims that are written in a 'consisting of' format and fully open claims that are drafted in a 'comprising' format."

[0039] "Homologous" as used herein means that the respective amino acid sequence or nucleotide sequence has a specified degree of identity with a reference amino acid sequence or nucleotide sequence. A homologous sequence is taken to include an amino acid sequence that is at least 75 %, 80 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 % or even 99% identical to the subject sequence, using the conventional sequence alignment tool Clustal W with default parameters. Typically, homologues will include the same active site residues as the subject amino acid sequence, though may include any number of conservative amino acid substitutions. "Identical" as used herein refers to an amino acid or nucleotide sequence identity to a reference sequence of 100 %.

[0040] The term "recombinant," when used in reference to a subject cell, nucleic acid, protein or vector, indicates that the subject has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, or express native genes at different levels or under different conditions than found in nature.

[0041] As used herein, the terms "transformed," "stably transformed," and "transgenic," used with reference to a cell means that the cell contains a non-native (e.g., heterologous) nucleic acid sequence integrated into its genome or carried as an episome that is maintained through multiple generations.

[0042] The term "fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy terminal deletion of one or more amino acids as compared to the native or wild-type protein but where the remaining amino acid sequence is identical to the corresponding positions in the amino acid sequence deduced from a full-length cDNA. Fragments are typically at least 50 amino acids in length.

[0043] The term "glycosylation" as used herein refers to the attachment of glycans to molecules, for example to proteins. Glycosylation may be an enzymatic reaction. The attachment formed may be through covalent bonds. Accordingly, a glycosylated polypeptide as used herein is a polypeptide to which a glycan is attached. The phrase "highly glycosylated" refers to a molecule such as an enzyme which is glycosylated at all or nearly all of the available glycosylation sites, for instance O-linked or N-linked glycosylation sites.

[0044] The term "glycan" as used herein refers to a polysaccharide or oligosaccharide, or the carbohydrate section of a glycoprotein or glycosylated polypeptide. Glycans may be homo- or heteropolymers of monosaccharide residues. They may be linear or branched molecules. Glycans typically contain at least three sugars and can be linear or branched. A glycan may include natural sugar residues (e.g., glucose, N-acetylglucosamine, N-acetyl neuraminic acid, galactose, mannose, fucose, hexose, arabinose, ribose, xylose, etc.) and/or modified sugars (e.g., 2'-fluororibose, 2'-deoxyribose, phosphomannose, 6' sulfo N-acetylglucosamine, etc.).

[0045] The term "O-glycans" as used herein refers to glycans that are generally found covalently linked to serine and threonine residues of mammalian glycoproteins.

[0046] O-glycans may be α -linked via an *N*-acetylgalactosamine (GalNAc) moiety to the - OH of serine or threonine by an O-glycosidic bond. Other linkages include α -linked O-fucose, β -linked O-xylose, α -linked O-mannose, β -linked O-GlcNAc (*N*-acetylglucosamine), α - or β -linked O-galactose, and α - or β -linked O-glucose glycans.

[0047] The term "sialylated" as used herein refers to molecules, in particular glycans that have been reacted with sialic acid or its derivatives.

[0048] The terms "binding affinity" or "affinity" as used herein indicate the strength of the binding between two molecules, in particular a ligand and a protein target. Binding affinities are influenced by non-covalent intermolecular interactions between the two molecules, such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and van-der-Waals forces.

[0049] An immune response as used herein relates to adaptive or innate immune response. The innate immune response refers to nonspecific defense mechanisms that are activated immediately or within hours of an antigen's appearance in the body. These mechanisms include physical barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body. The innate immune response is activated by chemical properties of the antigen. The adaptive immune response refers to antigen-specific immune responses. For this, the antigen first must be processed and recognized. Once an antigen has been recognized, the adaptive immune system creates a large number of immune cells specifically designed to attack that antigen.

[0050] As used herein, "immune tolerance" (or simply "tolerance") is the process by which the immune system does not attack an antigen. It occurs in three forms: central tolerance, peripheral tolerance and acquired tolerance. Tolerance can be either "natural" or "self-tolerance," where the body does not mount an immune response to self-antigens, or "induced tolerance", where tolerance to antigens can be created by manipulating the immune system.

Glycosylated Polypeptide

[0051] According to a first aspect the invention provides a glycosylated polypeptide comprising an amino acid sequence being at least 90 % identical to at least a fragment of human vWF (SEQ ID NO: 1), wherein said glycosylated polypeptide contains one or more clusters of O-glycosylation sites, wherein said clusters contain at least two O-glycosylation sites and one or more sialylated O-glycans, wherein the combined number of sialylated core 2 and extended core 1 O-glycans of the glycosylated polypeptide is higher than the number of sialylated core 2 and extended core 1 O-glycans of the human vWF or the fragment thereof and wherein the glycosylated polypeptide shows

[0052] The glycosylated polypeptide according to the invention is based on human VWF, i.e. contains an amino acid sequence identical or homologous to VWF or a fragment thereof..

[0053] Factor VIII in humans is coded by the F8 gene which comprises 187.000 base pairs and six exons. The transcribed mRNA has a length of 9.029 base pairs and is translated to a protein with 2.351 amino acids from which by a posttranslational modification 19 amino acids are removed. The FVIII molecule in humans is glycosylated on a 31 amino acid side chain (25 × N-glycosylation, 6 × O-glycosylation).

[0054] After translation, the amino acid chain is cleaved by specific proteases onto positions leading to the formation of a heavy chain with about 200 kDa and a light chain with about 80 kDa. The domain organization is typically characterized as A1-A2-B-A3-C1-C2. The light chain is a made-up of domains A3-C1-C2. The heavy chain is composed of the domains A1-A2-B. Heavy chains found in plasma have a heterogeneous composition with molecular weights varying from 90 to 200 kDa. The reason for this is the heterogeneity in its glycosylation, the existence of splice variants and the existence of proteolytic products such as the B domain depleted heavy chain A₁ A₂. The amino acid sequence of the full-length FVIII is identified by amino acids 20 to 2.351 of P00451 of SwissProt, July 21, 1986.

[0055] The human protein is preferably full-length FVIII identified by amino acids 20 to 2.351 of P00451 of SwissProt, July 21, 1986, a B-domain deleted FVIII or a FVIII protein in which a part of the B-domain has been replaced by a linker.

[0056] vWF is a multimeric adhesive glycoprotein present in the plasma of mammals, which has multiple physiological functions. During primary hemostasis, vWF acts as a mediator between specific receptors on the platelet surface and components of the extracellular matrix such as collagen. Moreover, vWF serves as a carrier and stabilizing protein for procoagulant Factor VIII. VWF is synthesized in endothelial cells and megakaryocytes as a 2813 amino acid precursor molecule. The precursor polypeptide, pre-pro-vWF, consists of a 22-residue signal peptide, a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma Von Willebrand Factor (Fischer et al., 1994). Full length vWF is identified by Uniprot entry P04275.

[0057] Upon secretion into plasma, vWF circulates in the form of various species with different

molecular sizes. These vWF molecules consist of oligo- and multimers of the mature subunit of 2050 amino acid residues. vWF can be usually found in plasma as multimers ranging in size approximately from 500 to 20.000 kDa (Furlan et al., 1996). The vWF in particular has an amino acid sequence of any of the sequences of Uniprot entry P04275. More preferably the vWF protein is identified by SEQ ID NO: 1.

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SLSCRPPMVKLVCADNLRAGLECKTKCQNYDLECMGCVSGCLCPPGMVRHENRCVALERCPC
FHQKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLKYLFPGECQYVLVQ
DYCGSNPGTFRILVGNKGCSHPSVKCKKRVITLVEGGEIELFDGEVNVKRPMKDETHFEVVESGRY
IILLGKALSVMWDRHLSISVVLKQTYQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDGNSWKV
SSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRILTSDVFQDCNKLVDPEPYLDVCIYDTCSCES
IGDCACFCDTIAAYAHVCAQHGVVTVRTATLCPQSCEERNLRENGYECWEYRNSCAPACQVTCQH
PEPLACFPVQCVGCHAHCPGKILDELLQTCVDPEDCPVEVAGRRFASGKKVTLNPSDPEHCQIC
HCDVNNLTCEACQEPGGLVVPPTDAPVSPTTLYVEDISEPPLHDFYCSRLLDLVFLLDGSSRLSEA
EFEVLKAFVVDMMERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRIASQVKYAGSQVAST
SEVLKYTLFQIFSKIDRPEASRITLLLMASQEPQRMRSNFVRYVQGLKKKKVIVIPVGIGPHANLK
QIRLIEKQAPENKAFVLSSVDELEQQRDEIVSYLCDLAPEAPPPTLPPDMAQVTVGPGLLGSTLG

PKRNSMVLDAFVLEGSCKIGEADFNRSKEFMEEVIQRMDVGQDSIHVTVLQYSYMTVEYPFSEA
QSKGDILQRVREIRYQGGNRTNTGLALRYLSDHSFLVSQGDREQAPNLVYMTGNPASDEIKRLPG
DIQVVPVIGVGNANVQELERIGWPNAPILIQDFETLPREAPDLVLQRCSSGEGQLIPTLSPAPDCS
QPLDVILLLDGSSSFASYFDEMKSFAKAFISKANIGPRLTQVSVLQYGSITTIDVPWNVPEKAH
LLSLVDVMQREGGPSQIGDALGFAVRYLTSEMHGARGPGASKAVVILVTDVSVDSVDAADAARSNR
VTVFPIGIGDRYDAAQLRILAGPAGDSNVVKLQRIEDLPTMVTLGNSFLHKLCSGFVRICMDEGDN
EKRPDGVWTLPDQCHTVTCQPDGQTLKSHRVNCDRGLRPSCPNSQSPVKVEETCGCRWTCPCVCT
GSSTRHIVTFDQNFKLGTGSCSYVLFQNKQDLEVLHNGACSPGARQGCMSIEVKHSALSVELH
SDMEVTVNGRLVSVPYVGGNMEVNVYGAIMEVRFNHLGHI FTFTPONNEFQLQLSPKTFASKTYG
LCGICDENGANDFMLRDGTVTTDWKTLLVQEWTVQRPQGTCQPILEEQCLVPDSSHCQVLLLPLFAE
CHKVLAPATFYAICQQDSCHQEQQVCEVIASIAHLCRTNGVCVDWRTPDFCAMSCPPSLVYNHCEHG
CPRHCDGNVSSCGDHPSEGCFPPDKVMLEGSCVPEEACTQCIGEDGVQHGFLEAWVPDHQPCQIC
TCLSGRKVNCTTQPCPTAKAPTCGLCEVARLRQNAQCCPEYECVCDPVSCDLPPVPHCERGLQPT
LTNPGECRPNFTACRKEECKRVSPSPCPLHRLPTLRKTQCCDEYECACNCVNSTVSCPLGYLAST
ATNDGCTTTTTCLPDKVCVHRSTIYPVGQFWEEGCDVCTCTDMEDAVMGLRVAQCSQKPCEDSCRS
GFTYVLHEGECCGRCLPSACEVVTGSPRGDSQSSWKSQVGSQWASPENPCLINECVRVKEEVFIQQR
NVSCPQLEVVPVCPSPGFQLSCKTSACCPSCRCERMEACMLNGTVIGPGKTMIDVCTTCRCMVQVGV
ISGFKLECRKTTNCPCLGYKEENNTGECCGRCLPTACTIQLRGGQIMTLKRDETLQDGCDFHCK
VNERGEYFWEKRVTCPPFDEHKCLAEGGKIMKIPGTCCDTCEEPECNDITARLQYVKVGSCKSEV
EVDIHYCQGGKASKAMYSIDINDVQDQSCCSPTRTEPMQVALHCTNGSVVYHEVLNAMECKCSPR
KCSK (SEQ ID NO: 1)
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[0058] The glycosylated polypeptide may for example contain a fragment of vWF as defined in WO 2015/185758 A2. As shown in WO 2015/185758 A2, the complex of FVIII and the vWF fragments as defined therein exhibit a reduced binding to phospholipid membranes compared to FVIII alone as well as a reduced binding to collagen III and heparin compared to the complex of FVIII and full-length vWF.

[0059] In this regard, the fragment of vWF is in particular a fragment starting with amino acid 1 of SEQ ID NO: 1. Amino acids 1 to 272 of SEQ ID NO: 1 comprise the FVIII binding domain of vWF.

[0060] The fragment of vWF preferably starting with amino acid 1 of SEQ ID NO: 1 preferably ends with an amino acid of SEQ ID NO: 1 in the range from 1142 to 1390.

[0061] The fragment more preferably ends with an amino acid of SEQ ID NO: 1 in the range from 1267 to 1390. More preferably, the vWF fragment ends with an amino acid of SEQ ID NO: 1 in the range from 1337 to 1390.

[0062] It is to be understood that the glycosylated polypeptide has an increased binding affinity compared to the mammalian protein or fragment defined by the amino acid sequence contained in the glycosylated peptide. Thus, if the glycosylated polypeptide comprises the amino acid sequence of a full-length mammalian protein, the glycosylated polypeptide has a higher affinity to SIGLECs compared to the full-length mammalian protein.

[0063] On the other hand, if the glycosylated polypeptide comprises a fragment of a mammalian protein defined by a subsequence of the mammalian protein, the glycosylated polypeptide has an increased binding affinity as compared to the identical fragment derived from the naturally occurring protein. For example, if the amino acid sequence in the glycosylated polypeptide is identical or homologous to a fragment of vWF, the glycosylated polypeptide according to the first aspect has an increased binding affinity to one or more SIGLECs as compared to the same fragment obtained from the fragmentation of plasma-derived vWF.

[0064] As shown in the examples, a glycan structure of vWF was determined that specifically binds to at least the SIGLECs SIG-5, SIG-7, SIG-8 and SIG-9 (see example 1). Thus, according to one embodiment of the first aspect, the one or more SIGLECs are selected from the group SIG-5, SIG-7, SIG-8 and SIG-9.

[0065] The inventors have surprisingly found that in human vWF the O-glycans are responsible for binding to SIG-5, SIG-7, SIG-8 and SIG-9. In contrast, the N-glycans do not show any binding to these SIGLECs (see example 2). This is particularly surprising, because so far it was rather the N-glycans that were shown to interact with SIGLECs (Lai et al., 2015).

[0066] The inventors further determined, that the O-glycans not only have to be sialylated for binding to SIG-5, SIG-7, SIG-8, SIG-9, but also a minimal percentage of core 2 glycans has to be present (see example 4).

[0067] Therefore, the SIGLEC binding and consequently, the reduced immune response is based on an increased number or percentage of sialylated core 2 O-glycans in the glycosylated protein as compared to the number of sialylated core 2 O-glycans of the mammalian protein or fragment thereof.

[0068] Due to structural similarities, it is assumed that sialylated extended core 1 O-glycans have the same effect as sialylated core 2 glycans. Thus, to increase the binding affinity to the SIGLECs defined above, preferably the combined number or percentage of sialylated core 2 and extended core 1 O-Glycans is increased.

[0069] Thus, according to one embodiment the number of sialylated core 2 and/or extended core 1 O-glycans of the glycosylated polypeptide is higher than the number of sialylated core 2 and/or extended core 1 O-glycans of the mammalian protein or fragment thereof. In this regard also the percentage of sialylated core 2 and/or extended core 1 O-glycans is increased as compared to the percentage of core 2 and/or extended core 1 O-glycans of the mammalian protein.

[0070] This means that the combined number of sialylated core 2 O-glycans and sialylated extended core 1 O-glycans of the glycosylated polypeptide is higher than the combined number of sialylated core 2 O-glycans and sialylated extended core 1 O-glycans of the mammalian protein or fragment thereof.

[0071] According to one embodiment, the number of sialylated core 2 O-glycans may be increased. In this regard also the percentage of sialylated core 2 O-glycans is increased as compared to the percentage of core 2 O-glycans of the mammalian protein.

[0072] The SIGLECs for which a binding was shown are involved in the immune response of humans and mice. SIGLECs have in common an N-terminal V-set Ig domain that binds sialic acid containing ligands, and a variable number of C2-set Ig domains that extend the ligand-binding side away from surface of the membrane.

[0073] In addition, many SIGLECs have cytoplasmic tyrosine motifs, including immunoreceptor tyrosine-based inhibitory motif (ITIM) and ITIM-like motifs, commonly found in co-receptors involved in regulation of cell signalling. Other SIGLECs do not contain tyrosine-motifs but contain a positively charged transmembrane spanning region that permits association with the adapter proteins. SIGLECs do not recognize danger associated molecular patterns (DAMPs) but instead determinants of "self".

[0074] SIGLECs bind to such sialylated self-ligands on the same cell in "cis" and on adjacent cells in "trans". The human SIGLECs are usually referred to as SIG-1 to SIG-14. The mouse SIGLECs SIG-E, SIG-F and SIG-G are orthologs of the human SIGLECs SIG-9, SIG-8 and SIG-10, respectively.

[0075] SIG-1 to SIG-4 are also referred to by the names Sialoadhesin, CD22, CD33 and MAG, respectively. CD22 and SIG-10 are located on B-cells, SIG-5 on neutrophils and monocytes, SIG-7 on NK-cells, SIG-8 on eosinophils, SIG-9 on monocytes, neutrophils and dendritic cells (Paulson et al., 2012).

[0076] SIGLECs bind to a variety of different glycan structures. Each of the SIGLECS SIG-2, SIG-5, SIG-7, SIG-8, SIG-9 and SIG-10 has a different glycan preference (Paulson et al., 2012). SIGLECs play a role in innate and adaptive immunity. In particular SIG-2 and SIG-10 that are located on B-cells of humans and mice.

[0077] According to Paulson et al., SIG-2 and SIG-10 appear to synergistically contribute to peripheral B-cell tolerance. Additionally, SIGLECs appear to act as inhibitory co-receptors for toll-like receptors (TLRs). In this regard, it was shown that cross-linking SIG-7 or SIG-9 to activation receptors results in inhibition of the cytolytic activity of NK-cells against tumor cells and the release of chemical mediators from mast cells, respectively.

[0078] Moreover, cross-linking of SIG-E (SIG-9) and SIG-11 by an immobilized anti-body results in inhibition of cytokine production in response to LPS in macrophages.

[0079] Of note, a topic expression of SIG-5 and SIG-9 in a macrophage cell line has been shown to inhibit the TNF-alpha production and enhance IL-10 production in response to peptidoglycan, ATR2 ligand, LPS and CpG. In addition, LPS induced SIG-E (SIG-9) expression in macrophages appears to effect the TLRsignalling. Also, sialylated pathogens dampen the immune response via SIGLECs. As an example, group B streptococcus expresses the Neu-Ac α -1 Gal β -1-4GlcNAc residue on the capsular polysaccharides and recruits SIGLEC-9 on neutrophils, resulting in suppression of microbicidal function of neutrophils.

[0080] Accordingly, without wanting to be bound to theory, it is believed that the binding to SIGLECS on antigen presenting cells (like e.g. dendritic cells) lead to down-regulation of pro-inflammatory and up-regulation of immunosuppressive receptors expression on the cell surface. Also, the binding leads to an enhanced production of anti-inflammatory cytokines, lowers the production of pro-inflammatory cytokines, and in consequence leads to the inhibition of T-cell proliferation and antibody production. Thus, binding of the SIGLECs SIG-5, SIG-7, SIG-8 and SIG-9 leads to a reduced immune response or increased immune tolerance when the glycosylated polypeptide is administered to a patient.

[0081] Thus, the glycosylated polypeptide according to the first aspect can also be defined as a glycosylated polypeptide comprising an amino acid sequence being at least 90 % identical to at least a fragment of human VWF wherein in comparison to the human VWF thereof:

- the immune response of a human to the glycosylated polypeptide is reduced; and/or
- the immune tolerance of human to the glycosylated polypeptide is increased.

[0082] The glycosylated polypeptide comprises an amino acid sequence being at least 90 % identical to at least a fragment of human VWF wherein said glycosylated polypeptide contains one or more sialylated O-glycans and wherein the combined number of sialylated core 2 O-glycans and sialylated extended core 1 O-glycans of the glycosylated polypeptide is higher than the combined number of sialylated core 2 O-glycans and sialylated extended core 1 O-

glycans of the human VWF or fragment thereof.

[0083] The glycosylated polypeptide comprises an amino acid sequence being at least 90 % identical to at least a fragment of a mammalian, preferably a human protein, wherein said glycosylated polypeptide contains one or more sialylated O-glycans and wherein the number of sialylated core 2 O-glycans of the glycosylated polypeptide is higher than the combined number of sialylated core 2 O-glycans of the mammalian protein or fragment thereof.

[0084] In order to couple the O-glycans to the amino acid sequence of the glycosylated polypeptide it contained one or more O-glycosylation sites. O-glycosylation sites of the glycosylated polypeptide can be the standard O-glycosylation sites serine (Ser), and threonine (Thr). However, also the binding of O-glycans to tyrosine (Tyr), hydroxyllysine (Hydroxy-Lys) or hydroxyproline (Hydroxy-Pro) has been described and is possible in the context of the invention. Thus, the one or more O-glycosylation sites in the glycosylated polypeptide may be selected from Ser, Thr, Tyr, Hydroxy-Lys and Hydroxy-Pro in any further possible O-glycosylation site. Preferably, the O-glycosylation sites are selected from Ser and Thr.

[0085] The standard glycosylation sites Ser and Thr generally show the highest occupation with O-glycans. Thus, according to one embodiment the one or more glycosylation sites are selected from Ser and Thr.

[0086] In the context of the invention for practical reasons the term "glycosylated polypeptide" is used in the singular form. Generally, the glycosylated polypeptide will occur in form of a composition of polypeptides of the same type. In this regard, glycosylated polypeptides in early form are a composition of glycosylated polypeptides having the same amino acid sequence, however, variances in the glycosylation. For example, not all of the individual molecules of the composition may be glycosylated to 100 percent. Moreover, differences in the glycans bound to a specific O-glycosylation site may arise. Accordingly, the present invention also relates to a composition comprising at least glycosylated polypeptide molecules of a first type, wherein the amino acid sequence of the protein molecules of the first type is identical or homologous to at least a fragment of a mammalian, preferably human protein and the protein molecules contain one or more glycosylation sites.

[0087] Preferably, the polypeptide contains one or more clusters of glycosylation sites. Although a single glycosylation site may be sufficient of SIGLEC binding, this assumed the formation of a cluster of O-glycosylation sites leads to an improved binding to SIGLECs. Cluster formation of glycosylation sites is often observed in mammalian proteins, examples are human IgA containing clustered O-glycans in the hinge region (cf. Franc et al., 2013) and human mucin (cf. Guzman-Aranguez and Argüeso, 2010).

[0088] In this regard, already two O-glycosylation sites in close proximities are considered an O-glycosylation cluster. Thus, the one or more cluster of O-glycosylation sites contain at least two O-glycosylation sites. The clusters of O-glycosylation sites may have different numbers of O-glycosylation sites. For example, the glycosylated polypeptide may contain one cluster with

two and a second cluster with three glycosylation sites. Between the O-glycosylation sites of a cluster also N-glycosylation sites may be present. Preferably there are no N-glycosylation sites in the O-glycosylation cluster.

[0089] Furthermore, one cluster may contain three O-glycosylation sites and the other four O-glycosylation sites. A number of three O-glycosylation sites results in three neighboring O-glycans that all may interact with the SIGLECs and therefore lead to an increased effect. According to one embodiment, the one or more clusters of O-glycosylation sites preferably contain at least three O-glycosylation sites.

[0090] In vWF, two clusters are present with four O-glycosylation sites each. Thus, preferably a polypeptide contains one or more clusters with at least four O-glycosylation sites. Presently, it is assumed that the higher the number of O-glycosylation sites, the higher is the binding affinity.

[0091] A cluster of O-glycosylation sites may be defined by two or more O-glycosylation sites within a short distance in the amino acid sequence. Those clusters are also referred to as "sequence clusters". However, due to the three-dimensional assembly of the glycosylated polypeptide, an O-glycosylation cluster may also include O-glycosylation sites that are in a further distance within the amino acid sequence, but after folding are located in close proximity. This latter type of clusters is also referred to as "folding cluster".

[0092] The vWF O-glycosylation cluster 2 contains 4 O-glycosylation sites within 20 amino acids, which are arranged as a beta turn. Accordingly, the distance of the O-glycans or O-glycosylation sites are 27.2 Å to 34.0 Å, leading to a mean distance of 6.8 Å to 8.5 Å. Thus, the mean distance of two O-glycosylation sites in a cluster may be in the range from 4.0 Å to 15.0 Å. Below 4.0 Å there may be a sterical hinderance of the O-glycans, in particular it may not be possible to glycosylate both O-glycosylation sites. Above the mean distance of two amino acids of more than 15.0 Å it is likely that there is no collaborative effect of the O-glycans. The collaborative effect is, e.g., an interaction with SIGLECs on the same cell. Preferably, the mean distance of two O-glycosylation sites in a cluster is in the range from 5.0 Å to 12.0 Å. More preferably, the mean distance of two O-glycosylation sites in a cluster is in the range from 6.0 Å to 9.0 Å.

[0093] Folding clusters may span amino acid sequences of over 100 amino acids. However, it is preferred that the spatial arrangement of the cluster does not exceed 80 Å. If the O-glycosylation sites are spaced apart more than 80 Å, it is assumed that the O-glycans do not exhibit a combined effect. The combined effect of the O-glycans in a cluster are the strongest, if the O-glycans are located within an area with a diameter of 50 Å. Thus, more preferably the spatial arrangement cluster does not exceed 50 Å.

[0094] According to one embodiment the one or more clusters, i.e. sequence clusters, contain at least one O-glycosylation site in ten amino acids. If the O-glycosylation sites are spread out further, it is believed that O-glycans bound to the O-glycosylation site cannot properly act

together on the same cell containing the SIGLECs.

[0095] Preferably, the one or more clusters contain at least one O-glycosylation site in four amino acids. With an average distance of the O-glycosylation sites of four amino acids there is a high chance that the O-glycosylation sites are also in close proximity after folding. The spatial proximity allows for a collaborative interaction of the glycans in the cluster with SIGLECs on the same cell.

[0096] More preferably, the one or more clusters contain at least one O-glycosylation site in three amino acids. As shown in the examples, the tested vWF peptide contains clusters with one O-glycosylation site in two amino acids. Thus, according to a preferred embodiment the one or more clusters contain at least one glycosylation site in two amino acids.

[0097] As shown in the examples, already one of such O-glycosylation clusters is enough for strong interaction with SIGLECs. Moreover, it is also shown by comparison of two vWF polypeptides that a higher number of clusters of O-glycosylation sites leads to an increased binding affinity of the peptide to SIGLECs, in particular to SIG-5, SIG-7, SIG-8 and SIG-9. Thus, the glycosylated polypeptide preferably comprises at least two glycosylation clusters, more preferably at least three glycosylation clusters.

[0098] Without wanting to be bound to theory, the binding affinity of the glycosylated polypeptide to the SIGLECs is higher the closer the clusters are located. In this regard, if two clusters are present, they are preferably separated by less than 100 amino acids. A distance of less than 100 amino acids allows a collaborative effect of the clusters of glycans in SIGLEC binding. More preferably, two clusters are separated by less than 50 amino acids. Most preferably two clusters are separated by less than 30 amino acids.

[0099] According to one embodiment the distance between any two neighboring clusters is less than 100 amino acids, preferably the distance between any two clusters within the glycosylated polypeptide is less than 50 amino acids. More preferably, the distance between any two neighboring clusters is less than 30 amino acids.

[0100] The glycosylated polypeptide preferably contains at least one additional cluster of O-glycosylation sites as compared to the human protein to which the sequence is homologous or identical.

[0101] As defined above, like all glycosylated polypeptides, the glycosylated polypeptide according to the invention represents a composition of glycosylated polypeptide molecules. These molecules exhibit a certain degree of heterogeneity within the glycosylation pattern, in particular not all of the glycosylation sites are necessarily occupied by O-glycans. The occupation by O-glycans is in particular dependent on the host cell in which the recommended glycosylated polypeptide is produced. Preferably, the host cell, i.e. expression system is chosen such that the percentage of occupation of the O-glycosylation sites is above 70%. If the occupation is below 70%, not enough O-glycans may be present for SIGLEC binding.

Preferably, more than 80% of the O-glycosylation sites are occupied by O-glycans. More preferably, more than 90% of the O-glycosylation sites are occupied with O-glycans. According to a preferred embodiment more than 95% of the O-glycosylation sites are occupied by O-glycans.

[0102] The composition of glycans attached to the glycosylated polypeptide depends on the method of production. The O-glycans may be natural or synthetic glycans. Natural O-glycans are for example glycans with the following core structure:

Core 1 O-glycan: Gal β 1 $\rightarrow$ 3GalNAc α 1 $\rightarrow$ Ser/Thr

Extended Core 1: O-glycan: Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3GalNAc α 1 $\rightarrow$ Ser/Thr

Core 2 O-glycan: Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 6)GalNAc α 1 $\rightarrow$ Ser/Thr

[0103] SIGLECs are known to bind to sialic acid. In conformity with this it is shown in the examples that desialylation abolished binding to the SIGLECs (see Example 3). Thus, it is confirmed that sialylation of the O-glycans is a pre-requisite for binding. Accordingly, a high percentage of sialylation of the O-glycans in the glycosylated polypeptide is preferred.

[0104] Accordingly, the O-glycans of the glycosylated polypeptide are preferably sialylated, i.e. contain at least one sialic acid as part of the glycan molecule.

[0105] The sialylated O-glycans may contain at least two sialic acids in alpha 2-3 glycosidic linkage. The sialylated O-glycans in the glycosylated polypeptide are in particular core 1 or core 2 O-glycans. An overview of the structures of core 1, extended core 1 and core 2 O-glycans is given below:

Sialylated Core 1: O-glycan: NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3GalNAc α 1 $\rightarrow$ Ser/Thr

Sialylated extended Core 1 O-glycan:

NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3GalNAc α 1 $\rightarrow$ Ser/Thr

Sialylated Core 2 O-glycan: NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3)GalNAc α 1 $\rightarrow$ Ser/Thr

[0106] It is possible that both core 1 and core 2 and/or extended core 1 O-glycans need to be present on the glycosylated polypeptide. According to one embodiment the glycosylated polypeptide comprises sialylated core 1 O-glycans as well as sialylated core 2 and/or extended core 1 O-glycans. As shown in example 4, the percentage of core 2 glycans of 2.5 % based on the total number of O-glycans is not enough for SIGLEC interaction. Thus, according to one embodiment of the glycosylated polypeptide the percentage of core 2 O-glycans based on the number of O-glycans is at least 5 %. In the same example it is shown that cluster 2 with a core

2 O-glycan percentage based on a number of O-glycans of 10.78 % leads to a strong interaction with the SIGLECs. Thus, according to a preferred embodiment the percentage of core 2 O-glycans based on the number of O-glycans in the glycosylated polypeptide is at least 8 %. More preferably, the percentage of core 2 O-glycans based on the number of O-glycans is at least 10 %.

[0107] In example 7, it was determined that about 80 % of the glycopeptide molecules of the recombinantly produced vWF peptides contain either a core 2 O-glycan or an extended core 1 glycan. Accordingly, the percentage of sialylated core 2 and/or extended core 1 O-glycans based on the total number of O-glycans is at least 20 %. Thus, according to one embodiment the concentration of core 2 and/or extended core 1 O-glycans based on the number of O-glycans in the glycosylated polypeptide is at least 15 %, more preferably at least 18 % and most preferably at least 20 %.

[0108] The number or percentage of core 2 O-glycans in the glycosylated polypeptide, in particular the number or percentage of individual molecules of the glycosylated polypeptide bearing a core 2 O-glycan may be increased by any of the following strategies:

One strategy is using the enzyme β 1,6-N-acetylglucosaminyltransferase. This enzyme is involved in the formation of core 2 O-glycans. Thus, an increase in core 2 bearing molecules and/or the number of core 2 O-glycan per polypeptide molecule can be obtained by expression of the glycosylated polypeptide in a cell line that overexpresses a β 1,6-N-acetylglucosaminyltransferase enzyme.

[0109] Another option is the expression of the glycosylated polypeptide in an expression cell line derived from a cancer cell line. Cancer cell lines are frequently shown to produce glycosylated proteins with a higher amount of core 2 O-glycans.

[0110] One strategy to increase the percentage of extended core 1 is using the enzyme β 1,3-N-acetylglucosaminyltransferase. This enzyme is involved in the formation of extended core 1 O-glycans. Thus, an increase in extended core 1 bearing molecules and/or the number of extended core 1 O-glycan per polypeptide molecule can be obtained by expression of the glycosylated polypeptide in a cell line that overexpresses a β 1,3-N-acetylglucosaminyltransferase.

[0111] The concentration of core 2 and/or extended core 1 sialylated O-glycans can be increased by chemical synthesis of glycans.

[0112] Accordingly, based on the teaching of the invention the skilled person can adapt the glycans to determine synthetic glycans with high binding affinity. According to a preferred embodiment the O-glycans are natural glycans.

[0113] According to one embodiment, at least a part of the sialylated core 2 O-glycans in the glycosylated polypeptide contains a sulfate group attached to galactose (Gal) or N-acetylglucosaminidase (GlcNAc) or fucose attached to GlcNAc in the core 2 type O-glycan,

which represents a high affinity binding ligand for SIGLECs 7, 8 and 9 (Paulson et al., 2012).

[0114] There are different ways known to the skilled person how to produce additional O-glycosylation sites. The glycosylated polypeptide may have an increased number of sialylated core 2 O-glycans due to an increased number of O-glycosylation sites as compared to the human protein or fragment thereof.

[0115] In this regard the glycosylated polypeptide may be a fusion protein, wherein a second amino acid sequence containing one or more O-glycosylation sites is covalently linked to the amino acid sequence identical or homologous to the human protein or fragment thereof (first amino acid sequence). The second amino acid sequence may be located N-terminal with respect to the first amino acid sequence Alternatively, the second amino acid sequence may be located C-terminal with respect to the first amino acid sequence. The glycosylated polypeptide may contain additional amino acid sequences both N- and C-terminal to the first amino acid sequence.

[0116] Accordingly, in such a fusion protein a second amino acid sequence and optionally further amino acid sequences may be present that mainly contain O-glycosylation sites, in particular O-glycosylation clusters. The cluster of O-glycosylation sites may be based on an amino acid sequence of a known mammalian, in particular human glycoprotein.

[0117] The second amino acid sequence may comprise one or more of the following O-glycosylation clusters:

WPPTXAPVXPTTXYVXXXSXPP (SEQ ID NO: 8),

VPPTDAPVSPPTLYVEDISEPP (SEQ ID NO: 9),

PPPTXPPXXAXVTVXPXXXVSTXXP (SEQ ID NO: 10),

PPPTLPPDMAQVTVGPGLLGVSTLGP (SEQ ID NO: 11),

VSSTSXXXXSTXPSXXXXAAXTXXTSSXXPPSXVXXXSXXXTTXXXX (SEQ ID NO: 12),

VSSTSNNLISTIPSDNLAAGTDDTSSLGPPSMPVHYDSQLDITLFGK (SEQ ID NO: 13),

XXXATTXPXXXXXXTXPXXX (SEQ ID NO: 14),

QFNATTIPENDIEKTDPWFA (SEQ ID NO: 15),

XXTTAATXXX (SEQ ID NO: 16),

LGTTAATELK (SEQ ID NO: 17),

XXPTPXXXSXXXXEAX (SEQ ID NO: 18),

QSPTPHGLSLSDLQEAK (SEQ ID NO: 19);

VXXXXXXXXXTXTSXXSPXXXXVXXSXXXXTXXAXX (SEQ ID NO: 20), and

VHIYQKDLFFTETSDGSPGHLDLVEGSLQGTGAIK (SEQ ID NO: 21).

[0118] In the sequences SEQ ID NO: 8, 10, 12, 14, 16, 18 and 20, X stands for any of the natural amino acids.

[0119] SEQ ID NO: 9 and 11 are found in vWF and SEQ ID NO: 13, 15, 17, 19 and 21 are derived from the B-domain of FVIII.

[0120] The second amino acid sequence may comprise one or more of the sequences selected from SEQ ID NO: 8, 10, 12, 14, 16, 18 and 20. The second amino acid sequence may contain combinations of the sequences. The second amino acid sequence preferably comprises multiple copies of one of the sequences SEQ ID NO: 8, 10, 12, 14, 16, 18 and 20. The second amino acid sequence may further comprise combinations of multiple copies of SEQ ID NO: 8, 10, 12, 14, 16, 18 and 20.

[0121] The second amino acid sequence may comprise one or more of the sequences selected from SEQ ID NO: 9, 11, 13, 15, 17, 19 and 21. The second amino acid sequence may contain combinations of the sequences. The second amino acid sequence preferably comprises multiple copies of one of the sequences of SEQ ID NO: 9, 11, 13, 15, 17, 19 and 21. The second amino acid sequence may further comprise combinations of multiple copies of SEQ ID NO: 9, 11, 13, 15, 17, 19 and 21.

[0122] According to a preferred embodiment, the second amino acid sequence contains one or more of copies of SEQ ID NO: 8.

[0123] Moreover, the second amino acid sequence may have a certain percentage of identity to the sequence of a naturally occurring glycosylated protein. The level of identity to a naturally occurring protein is preferable 80 %, more preferably at least 90 %.

[0124] Alternatively, the amino acid sequence of O-glycosylation sites in the second amino acid sequence may be fully synthetic. A fully synthetic amino acid sequence as used herein is a sequence not based on a known protein, in particular mammalian protein.

[0125] According to one embodiment the covalent linker connecting the second amino acid sequence to the amino acid sequence identical or homologous to the human protein or fragment thereof in the glycosylated polypeptide is selected from a peptide bond, a chemical linker, or a glycosidic bond. Chemical linkers eligible in this regard are:

- amine to amine linkers such as bismaleimidoethane, 1,8-bismaleimido-diethyleneglycol,
- amine to sulfhydryl linkers such as succinimidyl iodoacetate, N- α -maleimidoacetoxysuccinimide ester,

- carboxyl to amine linkers dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and
- sulfhydryl to carbohydrate linkers such as N- β -maleimidopropionic acid hydrazide, N- ϵ -maleimidocaproic acid hydrazide.

[0126] The linker of the fusion protein according to the invention may be formed by a spacer peptide sequence that separates the first and second amino acid sequence which define the fusion protein. The spacer peptide sequence may facilitate the correct folding of the individual protein or peptide parts and may make it more likely for the individual protein or peptide parts to retain their individual functional properties. Spacer peptide sequences may be inserted into fusion protein DNA sequences during the in-frame assembly of the individual DNA fragments that make up the complete fusion protein DNA sequence i.e. during overlapping PCR or DNA ligation.

[0127] A peptide bond has the advantage that the fully glycosylated polypeptide can be expressed at once as fusion protein.

[0128] The second amino acid sequence may be added to the first amino acid sequence by a chemical linker and therefore after expression of the protein.

[0129] As shown in the examples, in particular vWF (and fragments thereof) containing the O-glycosylation clusters 1 and/or 2 bind to SIGLECs.

[0130] According to one embodiment the sequence identity to human VWF (SEQ ID NO: 1) in the glycosylated polypeptide is at least 95% and more preferably at least 98%. According to a preferred embodiment the first amino acid sequence is at least 90 % identical to amino acids 1 to 505 of SEQ ID NO: 1.

[0131] The length of the second amino acid sequence is preferably in the range from 5 to 100 amino acids, more preferably from 10 to 80 amino acids, most preferably from 20 to 70 amino acids.

[0132] According to one embodiment the second amino acid is at least 98% homologous to amino acids 475 to 505 of SEQ ID NO: 1. Preferably, the second amino acids sequence is identical to amino acids 475 to 505 of SEQ ID NO: 1. According to a more preferred embodiment a second amino acid sequence is at least 98% homologous to two consecutive copies of amino acids 475 to 505 of SEQ ID NO: 1. Preferably, the second amino acid sequence is identical to two consecutive copies of amino acids 475 to 505 of SEQ ID NO: 1.

[0133] A representative fusion protein according to the invention is Seq12. Seq12 has the following amino acids sequence (SEQ ID NO: 2):

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPFGMVRHENRCVALERCPCFHHQKEY
APGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLKYLFPGECCQYVLVQDYCGSNPGTFRILV

GNKGCSHPSVKCKKRVITILVEGGEIELFDGEVNVKRPMDETHFEVVESGRYIILLGKALSVVWDRHLSIS
 VVLKQTYQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDFGNSWKVSSQCADTRKVPLDSSPATCHNNIMKQT
 MVDSSCRILTSDFVQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHKGKVVWTRTATLCP
 QSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKILDELLQTCVDPEDCPVCE
 VAGRRFASGKKVTLNPSDPEHCQICHCDVNVLTCEACQEPGGLVVPPTDAPVSPTTLYVEDISEPPLHQEPGG
 LVVPPTDAPVSPTTLYVEDISEPPLHQEPGGLVVPPTDAPVSPTTLYVEDISEPPLH

[0134] The following sequence (SEQ ID NO: 3) represents Seq12 with an additional 22 amino acid signal peptide (bold and underlined). An expression of this peptide provides a monomeric form of Seq12. The signal peptide is enzymatically cleaved off.

MI PARFAGVLLALALILPGTLCSLSCRPFMVKLVCPADNLRAEGLECTKTCQNYDLECMMSGCVSGCLCPPGM
 VRHENRCVALERCPCFHQGEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLKYLFPG
 ECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKKRVITILVEGGEIELFDGEVNVKRPMDETHFEVVESGR
 YIILLGKALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDFGNSWKVSSQCAD
 TRKVPLDSSPATCHNNIMKQTMVDSSCRILTSDFVQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAA
 YAHVCAQHKGKVVWTRTATLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPP
 GKILDELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQICHCDVNVLTCEACQEPGGLVVPPTDAPV
 SPTTLYVEDISEPPLHQEPGGLVVPPTDAPVSPTTLYVEDISEPPLHQEPGGLVVPPTDAPVSPTTLYVEDIS
 EPPLH (SEQ ID NO: 3)

[0135] A further representative fusion protein according to the invention is Pro-Seq12 including Seq12 and a propeptide (bold) with a signal peptide (bold and underlined). Pro-Seq12 is identified by SEQ ID NO: 4:

MI PARFAGVLLALALILPGTLCAEAGTRGRSSSTARCSLFGSDFVNTFDGSMYSFAGYCSYLLAGGCQKRFSII
 GDFQNGKRVSLSVYLGEFFDIHLFVNGTVTQGDQRVSMFYASKGLYLETEAGYKLSGEAYGFVARIDGSGNF
 QVLLSDRYFNKTCGLCGNFNI FAEDDFMTQEGTLTSDPYDFANSWALSSGEQWCERASPPSSSCNISSGEMQK
 GLWEQCQLLKSTSVFARCHPLVDPEPFVALCEKTLCECAGGLECACPALLEYARTCAQEGMVLYGWDHSACS
 PFCPAGMEYRQCVSPCARTCQSLHINEMCQERCVDGCSCEPQGLLDEGLCVESTECPCVHSGKRYPPGTSLSR
 DCNTCICRNSQWICSNEECPEGELVTGQSHFCSFDNRYFTFSGICQYLLARDCQDHSFSIVIETVQCADDRDA
 VCTRSVTVRLPGLHNSLVKLKHGAGVAMDGDVQLPLLKGLDLRIQHTVTASVRLSYGEDLQMDWDGRGRLLVK
 LSPVYAGKTCGLCGNYNGNQDDFLTPSGLAEPREDFGNAWKLGDCQDLQKQHS DPCALNPRMTRFSEEAC
 AVLTSPTFEACHRAVSPLPYLRNCRYDVCSCSDGRECLCGALASYAAACAGRVRVAWREPGRCCELNCPKGQV
 YLQCGTPCNLTCSRSLSPDEECNEACLEGCFCPPGLYMDERGDVCPKAQCPCYDGEIFQPEDIFSDHHTMCY
CEDGFMHCTMSGVPGSLLPDAVLSSPLSHRSKRSLSCRPFMVKLVCPADNLRAEGLECTKTCQNYDLECMMSG
 CVSGCLCPPGMVRHENRCVALERCPCFHQGEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYL
 TFDGLKYLFPGEQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKKRVITILVEGGEIELFDGEVNVKRPMD
 ETHFEVVESGRYIILLGKALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDFG
 NSWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRILTSDFVQDCNKLVDPEPYLDVCIYDTCSCESIG
 DCACFCDTIAAYAHVCAQHKGKVVWTRTATLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQ
 CVEGCHAHCPPGKILDELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQICHCDVNVLTCEACQEPG
 GLVVPPTDAPVSPTTLYVEDISEPPLHQEPGGLVVPPTDAPVSPTTLYVEDISEPPLHQEPGGLVVPPTDAPV
 SPTTLYVEDISEPPLH (SEQ ID NO: 4)

[0136] Expression of Pro-Seq12 results in the formation of dimers. The peptide dimers remain intact also after cleavage of the propeptide.

[0137] According to one embodiment the glycosylated polypeptides contains a first amino acid sequence that is at least 98% identical to amino acids 1 to 505 of SEQ ID NO: 1. The second amino acid sequence is at least 98% homologous to two consecutive copies of amino acids 475 to 505 of SEQ ID NO: 1.

[0138] According to one embodiment the glycosylated polypeptide is produced by expression in a human cell-line. Generally, any human cell-line is suitable for expression of the glycosylated polypeptide. A favourable glycosylation peptide is particularly obtained with HEK cell lines.

[0139] Examples of HEK cell-lines for production of the glycosylated polypeptide are HEK 293 F, Flp-In[™]-293 (Invitrogen, R75007), 293 (ATCC[®] CRL-1573), 293 EBNA, 293 H (ThermoScientific 11631017), 293S, 293T (ATCC[®] CRL-3216[™]), 293T/17 (ATCC[®] CRL11268[™]), 293T/17 SF (ATCC[®] ACS4500[™]), HEK 293 STF (ATCC[®] CRL 3249[™]), HEK-293.2sus (ATCC[®] CRL-1573[™]). A preferred cell line for production of the polypeptide is the HEK 293 F cell line.

[0140] Other cell lines suitable as host cells for expression include cell lines derived from human myeloid leukaemia cells. Specific examples of host cells are K562, NM-F9, NM-D4, NM-H9D8, NM-H9D8-E6, NM H9D8- E6Q12, GT-2X, GT-5s and cells derived from anyone of said host cells. K562 is a human myeloid leukemia cell line present in the American Type Culture Collection (ATCC CCL-243). The remaining cell lines are derived from K562 cells and have been selected for specific glycosylation features.

[0141] According to an alternative embodiment the one or more glycosylation sites are located within the amino acid sequence homologous or identical to the mammalian protein or fragment thereof. It is to be understood that O-glycosylation sites that are not present in the amino acid sequence of the mammalian protein or fragment are found within the homologous or identical amino acid sequence within the glycosylated polypeptide.

[0142] The one or more O-glycosylation sites within the amino acid sequence homologous or identical to the mammalian protein or fragment thereof may be inserted within the sequence. Alternatively, the one or more O-glycosylation sites may replace amino acids of the mammalian protein. An amino acid replacement is preferred as it does not change the size of the polypeptide chain and therefore is less likely to influence the three-dimensional structure of the protein.

[0143] The one or more O-glycosylation sites within the amino acid sequence are preferably in parts of the sequence that do not form binding sites or active centers of the protein. Moreover,

the one or more O-glycosylation sites are preferably added in an amino acid position that will be exposed to the surface of the folded protein. In order to achieve the least influence on the activity or integrity of the protein, the one or more O-glycosylation sites may be added to a flexible loop of the protein.

[0144] In case of FVIII proteins, the one or more O-glycosylation sites are preferably added in the position of or replacing the B-domain.

[0145] In one embodiment, the glycosylated polypeptide is modified by attachment with one or more biocompatible polymers to improve, e.g., half-life or stability. Suitable biocompatible polymers include polyalkylene oxides such as, without limitation, polyethylene glycol (PEG), dextrans, colominic acids or other carbohydrate based polymers, polymers of amino acids, biotin derivatives, polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-malic acid anhydride, polyoxazoline, polyacryloylmorpholine, heparin, albumin, celluloses, hydrolysates of chitosan, starches such as hydroxyethyl-starches and hydroxy propyl-starches, glycogen, agaroses and derivatives thereof, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrolysates, other bio-polymers and any equivalents thereof. In one embodiment, the polymer is polyethylene glycol (PEG). In another embodiment, the polymer is methoxypolyethylene glycol (mPEG). Other useful polyalkylene glycol compounds are polypropylene glycols (PPG), polybutylene glycols (PBG), PEG-glycidyl ethers (Epoxy-PEG), PEG-oxycarbonylimidazole (CDI-PEG), branched polyethylene glycols, linear polyethylene glycols, forked polyethylene glycols and multi-armed or "super branched" polyethylene glycols (star-PEG). The biocompatible polymer is preferably connected to the polypeptide by one of the following residues -SH, OH, -COOH.

[0146] According to one embodiment the glycosylated polypeptide is able to form dimers or multimers. The formation of dimers and in particular multimers increases the number of O-glycans or O-glycan clusters in close proximity. Thus, more O-glycans may interact with SIGLECs on one cell. Moreover, the O-glycans can interact with several SIGLEC expressing cells that are located closely together, thereby increasing the immune tolerance.

[0147] Multimerization may be the result of a multimerization domain in the amino acid sequence of the mammalian protein on which the glycosylated polypeptide is based. Alternatively, multimers of the glycosylated polypeptide can be formed by introducing multimerization domains into amino acid sequence of the glycosylated polypeptide.

[0148] An example of a fusion protein according to the invention that forms multimers is the Pro-Seq12-Mult including Seq12 and a propeptide (bold) with a signal peptide (bold and underlined) as well as a multimerization sequence, the "cystein knot domain" of vWF (underlined). Pro-Seq12-Mult is identified by SEQ ID NO: 5:

MI**PARFAGVLLALALILPGTLC****AEGTRGRSSTARCSLFGSDFVNTFDGSMYSFAGYCSYLLAGGCQKRSFSII**
GD**FQNGKRVSLSVYLGEFFDIHLFVNGTVTQGDQRVSMFYASKGLYLETEAGYYKLSGEAYGFVARIDGSGNF**
QVLLSDRYFNKTCGLCGNFNI**FAEDDFMTQEGTLTSDPYDFANSWALSSGEQWCERASPPSSSCNISSGEMQK**
GLWEQCQLLKSTSVFARCHPLVDPEPFVALCEKTLCECAGGLECACPALLEYARTCAQEGMVLYGWTDSACS

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 P V C P A G M E Y R Q C V S P C A R T C Q S L H I N E M C Q E R C V D G C S C P E G Q L L D E G L C V E S T E C P C V H S G K R Y P P G T S L S R
 D C N T C I C R N S Q W I C S N E E C P G E C L V T G Q S H F K S F D N R Y F T F S G I C Q Y L L A R D C Q D H S F S I V I E T V Q C A D D R D A
 V C T R S V T V R L P G L H N S L V K L K H G A G V A M D G Q D V Q L P L L K G D L R I Q H T V T A S V R L S Y G E D L Q M D W D G R G R L L V K
 L S P V Y A G K T C G L C G N Y N G N Q G D D F L T P S G L A E P R V E D F G N A W K L H G D C Q D L Q K Q H S D P C A L N P R M T R F S E E A C
 A V L T S P T F E A C H R A V S P L P Y L R N C R Y D V C S C S D G R E C L C G A L A S Y A A A C A G R G V R V A W R E P G R C E L N C P K G Q V

 Y L Q C G T P C N L T C R S L S Y P D E E C N E A C L E G C F C P P G L Y M D E R G D C V P K A Q C P C Y Y D G E I F Q P E D I F S D H H T M C Y
 C E D G F M H C T M S G V P G S L L P D A V L S S P L S H R S K R S L S C R P P M V K L V C P A D N L R A E G L E C T K T C Q N Y D L E C M S M G
 C V S G C L C P P G M V R H E N R C V A L E R C P C F H Q G K E Y A P G E T V K I G C N T C V C Q D R K W N C T D H V C D A T C S T I G M A H Y L
 T F D G L K Y L F P G E C Q Y V L V Q D Y C G S N P G T F R I L V G N K G C S H P S V K C K K R V T I L V E G G E I E L F D G E V N V K R P M K D
 E T H F E V V E S G R Y I I L L G K A L S V V W D R H L S I S V V L K Q T Y Q E K V C G L C G N F D G I Q N N D L T S S N L Q V E E D F V D F G
 N S W K V S S Q C A D T R K V P L D S S P A T C H N N I M K Q T M V D S S C R I L T S D V F Q D C N K L V D P E P Y L D V C I Y D T C S C E S I G
 D C A C F C D T I A A Y A H V C A Q H G K V V T W R T A T L C P Q S C E E R N L R E N G Y E C E W R Y N S C A P A C Q V T C Q H P E P L A C P V Q
 C V E G C H A H C P P G K I L D E L L Q T C V D P E D C P V C E V A G R R F A S G K K V T L N P S D P E H C Q I C H C D V V N L T C E A C Q E P G
 G L V V P P T D A P V S P T T L Y V E D I S E P P L H Q E P G G L V V P P T D A P V S P T T L Y V E D I S E P P L H Q E P G G L V V P P T D A P V
 P T T L Y V E D I S E P P L H E E P E C N D I T A R L Q Y V K V G S C K S E V E V D I H Y C Q G K C A S K A M Y S I D I N D V Q D Q C S C S P T
R T E P M Q V A L H C T N G S V V Y H E V L N A M E C K C S P R K C S K

[0149] Alternatively, multimerization of the glycosylated polypeptide may be obtained by binding to a polymer or a liposome.

Use of a glycosylated polypeptide

[0150] As defined above, the inventors have found that a protein with a glycan composition including sialylated core 2 O-glycans and/or sialylated extended core 1 O-glycans interacts with defined SIGLECs and therefore influences cells of the immune system of mammals. In particular a glycosylated polypeptide exhibits a reduced immune response. Therefore, such glycosylated polypeptide if administered with a second protein can influence the immune response of a patient to the second protein. Therefore, a glycosylated polypeptide with sialylated core 2 O-glycans and/or sialylated extended core 1 O-glycans can be used to modify, in particular reduce the immune response of a patient to a protein, in particular a therapeutic protein in combined administration.

[0151] Thus, according to a second aspect the invention relates to the use of a human VWF containing one or more sialylated O-glycans and exhibiting binding to one or more SIGLECs, selected from SIG-5, SIG-7, SIG-8, and SIG-9 for reducing the immune response of a second protein or increasing the immune tolerance of a patient to a second protein, wherein the second protein is in particular a therapeutic protein.

[0152] Preferably the glycosylated polypeptide used for reducing the immune response comprises sialylated core 2 O-glycans and/or sialylated extended core 1 O-glycans. More preferably, the glycosylated polypeptide is defined as the glycosylated polypeptide according to

the first aspect.

Composition and protein complex

[0153] Accordingly, the concept according to the invention, namely the reduced immune response of a human protein by e.g. addition of one or more sialylated core 2 O-glycans cannot only be achieved by the preparation of a fusion protein or by insertion or replacement of amino acids by O-glycosylation sites, but also by adding an additional polypeptide as described in the use according to the second aspect. This leads to the formation of a composition of the glycosylated polypeptide and a second polypeptide, the immune response of which is to be reduced.

[0154] Accordingly, in a third aspect the present invention also relates to a composition comprising a first and a second polypeptide, wherein the first polypeptide is a glycosylated polypeptide according to the first aspect and the second polypeptide contains an amino acid sequence homologous or identical to a second mammalian, in particular human protein, wherein compared to the second polypeptide the composition has an increased binding affinity to a SIGLEC selected from one or more SIGLECs, selected from SIG-5, SIG-7, SIG-8, and SIG-9.

[0155] It is also possible to provide a binding partner to a polypeptide, which has increased binding affinity to a SIGLEC, so that the complex of the two polypeptides has an increased binding affinity to the SIGLEC as compared to the polypeptide.

[0156] Thus, according to a third aspect, the invention provides a composition comprising a first and a second polypeptide, wherein the first polypeptide is a glycosylated polypeptide according to the first aspect and the second polypeptide contains an amino acid sequence homologous or identical to a second mammalian, in particular human protein, wherein compared to the second polypeptide:

- the composition has an increased binding affinity to a SIGLEC selected from ; SIG-5, SIG-7, SIG-8, and SIG-9 and/or
- the immune response of a human to the complex is reduced; and/or
- the immune tolerance of a human to the complex is increased.

[0157] The second human protein is preferably a human blood protein. The human blood protein may be a human blood clotting factor, a transport protein, a protease inhibitor, an immunoglobulin, a cell related plasma protein, an apolipoprotein, a complement factor, a growth factor, an antiangiogenic protein, a highly glycosylated protein, a blood factor or another human blood protein.

[0158] The human blood clotting factor is in particular selected from the group consisting of fibrinogen, fibrin monomer, prothrombin, thrombin, FV/FVa, FX/FXa, FIX/FIXa, FVII/FVIIa, FVIII/FVIIIa, FXI/FXIa, FXII/FXIIa, FXIII/FXIIIa, von Willebrand factor, and ADAMTS13.

[0159] The transport protein may be selected from albumin, transferrin, ceruloplasmin, haptoglobin, hemoglobin, and hemopexin.

[0160] Possible protease inhibitors are, e.g., β -antithrombin, α -antithrombin, oxidized-antithrombin, 2-macroglobulin, C1-inhibitor, tissue factor pathway inhibitor (TFPI), heparin cofactor II, protein C inhibitor (PAI-3), Protein C, Protein S, and Protein Z.

[0161] Examples of immunoglobulins such as polyclonal antibodies (IgG), monoclonal antibodies, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2, IgM, IgE, IgD, and Bence Jones protein.

[0162] The cell related plasma protein may be for example fibronectin, thromboglobulin, platelet factor 4. Examples of apolipoproteins are apo A-I, apo A-II, and apo E.

[0163] Complement factors according to the invention are e.g. Factor B, Factor D, Factor H, Factor I, C3b-Inactivator, properdin, C4-binding protein etc.

[0164] Examples of growth factors include Platelet derived growth factor (PDGF), Epidermal growth factor (EGF), Transforming growth factor alpha (TGF- α), Transforming growth factor beta (TGF- β), Fibroblast growth factor (FGF) and Hepatocyte growth factor.

[0165] Antiangiogenic proteins include latent-antithrombin, prelatent-antithrombin, oxidized-antithrombin and plasminogen.

[0166] Examples of highly glycosylated proteins are alfa-1-acid glycoprotein, antichymotrypsin, inter- α -trypsin inhibitor, α -2-HS glycoprotein, C-reactive protein, Blood factors may be, e.g., such as erythropoietin, interferon, tumor factors, tPA, gCSF.

[0167] Other human blood proteins include histidine-rich glycoprotein, mannan binding lectin, C4-binding protein, fibronectin, GC-globulin, plasminogen/plasmin, α -1 microglobulin, C-reactive protein.

[0168] The second human protein is in particular selected from FVIII, FVII/FVIIa, FIX, ADAMTS13.

[0169] The composition according to the third aspect is in particular a protein complex of the first and second polypeptide.

[0170] The second polypeptide contains an amino acid sequence identical to a mammalian, in particular human protein. In order to reduce the immune response of a human to the second polypeptide, a first polypeptide forms a complex with the second polypeptide.

[0171] For protein complex formation, the first polypeptide in particular comprises a binding domain, that allows a binding to the second polypeptide and a glycosylation domain. The glycosylation domain in particular comprises one or more O-glycosylation sites, preferably an O-glycosylation cluster.

[0172] According to one embodiment, the second polypeptide is a FVIII protein, and the first polypeptide comprises the FVIII binding domain of vWF and one or more O-glycosylation sites to which sialylated core 2 O-glycans are bound.

[0173] A preferred example of the composition is a protein complex of a FVIII protein with an amino acid sequence 95% identical to the sequence identified by amino acids 20 to 2.351 of P00451 and a first polypeptide as a binding partner comprising amino acid sequence at least 95% identical to amino acids 1 to 172 of SEQ ID NO: 1.

[0174] According to one embodiment of the protein complex of the third aspect, the first polypeptide is a polypeptide according to the first aspect.

[0175] According to a further embodiment, the second polypeptide may for example be selected from FVIII, FVII, FIX and ADAMTS13.

[0176] In one embodiment of the third aspect, the first polypeptide comprises at least a fragment of human vWF and a second polypeptide is a FVIII protein, in particular a full length FVIII protein, a B-domain deleted FVIII protein or a FVIII protein in which part of the B-domain has been replaced by a linker. According to one embodiment the first polypeptide is defined by amino acids 1 to 505 of SEQ ID NO: 1, which was produced in HEK-cells, in particular HEK 293F-cells.

[0177] According to a further embodiment the first polypeptide is defined by amino acid 1 to 505 of SEQ ID NO: 1 and one copy of amino acids 475 to 505 of SEQ ID NO: 1. Moreover, the first polypeptide may be defined by an amino acid sequence selected from SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5.

[0178] As shown in the examples, a protein with an improved binding affinity to SIGLECs, in particular SIG-5, SIG-7, SIG-8 and/or SIG-9, can be produced with the cell-line HEK 293F. Thus, according to a further embodiment of the protein complex, the first and second polypeptides are produced by recommended expression in a human cell-line, preferably a HEK cell-line. Examples of HEK cell-lines for production of the glycosylated polypeptide are HEK 293 F, Flp-InTM-293, 293, 293 EBNA, 293 H, 293S, 293T, 293T/17, 293T/17 SF, HEK 293 STF, and HEK-293.2sus. A preferred cell line for production of the polypeptide is the HEK 293 F cell line.

[0179] The first and second polypeptide can be produced by separate recombinant expression and joined afterwards. Alternatively, the first and second polypeptide are recombinantly expressed in the same cell. For this, the first and second polypeptide may be encoded by the

same vector or by two different vectors.

Polynucleotide

[0180] According to a fourth aspect, the invention provides an isolated polynucleotide that comprises a nucleic acid sequence encoding a glycosylated polypeptide according to the first aspect of the invention.

[0181] The isolated polynucleotide may be a DNA molecule or an RNA molecule. The isolated polynucleotide is preferably a DNA molecule, in particular a cDNA molecule. The techniques used to isolate or clone a polynucleotide encoding a peptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the polynucleotides from such genomic DNA can be effected, e.g., by using the well-known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features (see, e.g., Innis et al., 1990) PCR: A Guide to Methods and Application, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA) may be used.

[0182] The isolated polynucleotide may be a DNA molecule encoding a glycosylated polypeptide with an amino acid sequence similar or identical to a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5.

[0183] In particular, the isolated polynucleotide may be a DNA molecule encoding a glycosylated polypeptide having an amino acid sequence with an identity of at least 90 %, preferably at least 95 %, more preferably at least 98 %, most preferably 100 % to SEQ ID NO: 2. Moreover, the isolated polynucleotide may be a DNA molecule encoding a glycosylated polypeptide having an amino acid sequence with an identity of at least 90 %, preferably at least 95 %, more preferably at least 98 %, most preferably 100 % to SEQ ID NO: 3. According to one embodiment the isolated polynucleotide is a DNA molecule encoding a glycosylated polypeptide having an amino acid sequence with an identity of at least 90 %, preferably at least 95 %, more preferably at least 98 %, most preferably 100 % to SEQ ID NO: 4. According to one embodiment, the isolated polynucleotide is a DNA molecule encoding a glycosylated polypeptide having an amino acid sequence with an identity of at least 90 %, preferably at least 95 %, more preferably at least 98 %, most preferably 100 % to SEQ ID NO: 5.

Expression vector

[0184] In a fifth aspect the invention also relates to an expression vector comprising a polynucleotide according to the fourth aspect of the invention.

[0185] The expression vector further preferably comprises control elements such as a promoter, and transcriptional and translational stop signals. The polynucleotide according to the fourth aspect and of the control elements may be joined together to produce a recombinant expression vector that may include one or more restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. The polynucleotide may be inserted into an appropriate expression vector for expression. In creating the expression vector, the coding sequence is located in the expression vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0186] The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide of the fourth aspect of the invention. The choice of the expression vector will typically depend on the compatibility of the expression vector with the host cell into which the expression vector is to be introduced. The expression vectors may be a linear or closed circular plasmid.

[0187] The expression vector is preferably adapted to expression in mammalian cells. The expression vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate in vivo.

[0188] The vector is preferably one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. For integration into the host cell genome, the expression vector may rely on any other element of the expression vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location in the chromosome.

[0189] The vectors of the present invention preferably contain one or more (e.g., several) selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

[0190] The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook et al., 1989, supra).

[0191] According to one embodiment the vector backbone of the vector according to the fifth

aspect is selected from pCDNA3, pCDNA3.1, pCDNA4, pCDNA5, pCDNA6, pCEP4, pCEP-puro, pCET1019, pCMV, pEF1, pEF4, pEF5, pEF6, pExchange, pEXPR, pIRES, and pSCAS.

[0192] The vector according to the fifth aspect may be transiently or intransiently transformed into a host cell. The host cell may any of the cells listed above. Preferably the host cell is HEK 293F.

Medical use and method of treatment

[0193] As described above, the glycosylated polypeptide and the composition, in particular protein complex according to the invention have the advantage of a reduced immune response in patients, in particular human patients. Thus, a glycosylated polypeptide and a protein complex are in particular useful as active ingredients for medical treatment.

[0194] According to a sixth aspect, the invention provides a glycosylated polypeptide defined according to the first aspect for use in medical treatment. As an alternative, according to the sixth aspect the invention provides a composition defined according to the third aspect for use medical treatment. Preferably, the medical treatment is prevention of a bleeding disorder.

[0195] As used herein "bleeding disorder" refers to a disease or condition that impairs normal hemostasis. The bleeding disorder can be, for example, Hemophilia A, Hemophilia B, Factor VIII deficiency, Factor XI deficiency, von Willebrand Disease, Glanzmann's Thrombasthenia, Bernard Soulier Syndrome, idiopathic thrombocytopenic purpura, intracerebral hemorrhage, trauma, traumatic brain injury, and the like.

[0196] As used herein, "hemophilia" refers to a group of bleeding disorders associated with increased blood clot formation time as compared to blood clot formation time in healthy individuals without hemophilia. "Hemophilia" refers to both Hemophilia A, which is a disorder that leads to the production of defective Factor VIII, and Hemophilia B, which is a disorder that leads to the production of defective Factor IX.

[0197] The bleeding disorder is preferably haemophilia. The treatment may for example be the haemophilia treatment of Previously untreated patients (PUPS) () or an immune tolerance induction (ITI) treatment.

[0198] According to an alternative embodiment of the third aspect, the invention provides a protein complex defined according to the second aspect for use in the treatment or prevention of a bleeding disorder.

[0199] The treatment preferably comprises administering to a patient an effective amount of the glycosylated polypeptide or composition, in particular a protein complex.

[0200] The glycosylated polypeptide or composition, in particular protein complex, described

herein can be administered alone or in the form of pharmaceutical compositions. Pharmaceutical compositions according to the invention may comprise an effective amount of the conjugates formulated with at least one pharmaceutically acceptable carrier. Pharmaceutical compositions of the embodiments can be prepared and administered to a subject by any methods well known in the art of pharmacy. See, e. g, Goodman & Gilman's The Pharmacological Basis of Therapeutics, Hardman et al., eds., McGraw-Hill Professional (10th ed., 2001); Remington: The Science and Practice of Pharmacy, Gennaro, ed., Lippincott Williams & Wilkins (20th ed., 2003); and Pharmaceutical Dosage Forms and Drug Delivery Systems, Ansel et al. (eds), Lippincott Williams & Wilkins (7th ed., 1999). In addition, the pharmaceutical compositions of the embodiments may also be formulated to include other medically useful drugs or biological agents. The pharmaceutical composition typically comprises a therapeutically effective amount of the glycosylated polypeptide or protein complex combined with a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier is any carrier known or established in the art. Exemplary pharmaceutically acceptable carriers include sterile pyrogen-free water and sterile pyrogen-free saline solution. Other forms of pharmaceutically acceptable carriers that can be utilized for the present embodiments include binders, disintegrants, surfactants, absorption accelerators, moisture retention agents, absorbers, lubricants, fillers, extenders, moisture imparting agents, preservatives, stabilizers, emulsifiers, solubilising agents, salts which control osmotic pressure, diluting agents such as buffers and excipients usually used depending on the use form of the formulation. These are optionally selected and used depending on the unit dosage of the resulting formulation.

[0201] For in vivo applications, the glycosylated polypeptide, protein complex or pharmaceutical composition can be administered to the patient by any customary administration route, e.g., orally, parenterally or by inhalation. Parenteral administration includes intravenous injection, subcutaneous injection, intraperitoneal injection, intramuscular injection and intraperitoneal injection, liquid agents, suspensions, emulsions and dripping agents. For parenteral administration the glycosylated polypeptide, protein complex or pharmaceutical composition should be an injectable agent such as a liquid agent or a suspension.

[0202] In other embodiments, the glycosylated polypeptide, protein complex or pharmaceutical composition is administered orally to a patient. In these embodiments, a form of the drug includes solid formulations such as tablets, coated tablets, powdered agents, granules, capsules and pills, liquid formulations such as liquid agents (e.g., eye drops, nose drops), suspension, emulsion and syrup, inhales such as aerosol agents, atomizers and nebulizers, and liposome inclusion agents. In still some other embodiments, the glycosylated polypeptide, protein complex or pharmaceutical composition is administered by inhalation to the respiratory tract of a patient to target the trachea and/or the lung of a subject.

[0203] According to one embodiment of the sixth aspect, the glycosylated polypeptide or composition, in particular protein complex for use, the use comprises an intravenous or non-intravenous injection. The non-intravenous injection preferably is a subcutaneous injection.

[0204] The invention will further be described by the following non-limiting examples.

EXAMPLES

Example 1 - Binding von full length Willebrand factor (vWF) to SIGLECs:

1.1. Experimental Procedure

[0205] Recombinant SIG-2, SIG-5, SIG-7, SIG-F (mouse equivalent of human SIG-8), SIG-9 and SIG-10 were obtained as Fc-fusion proteins from R&D Systems. First, Protein A (SERVA Feinbiochemica GmbH & Co) was coated on a plate in a concentration 0.5 µg/well, at 4 °C, over night (O/N). After blocking and washing steps with washing buffer (20 mM HEPES, 125 mM NaCl, 1 mM EDTA, 1% BSA), Fc-fusion SIGLECs or control antibodies, were bound to protein A by 1h incubation at 37°C at 5 µg/ml concentration. Anti vWF-pAb (Dako, #A0082) was immobilized as a positive control and Anti-Chicken IgY (Sigma Aldrich, # C2288) as a negative control via antibody Fc part.

[0206] Plasma derived VWF (pdVWF), was biotinylated using EZ-Link™ Sulfo-NHS-Biotin biotinylation kit (Thermo Fisher Scientific). Concentration series of biotinylated, vWF was applied into the wells at concentrations of 0 to 0.8 µg/mL. After five washing steps with washing buffer, the HRP-coupled streptavidin (Thermo Fisher Scientific, #31001) was added to the wells and incubated 1h at 37°C with subsequent five washing steps.

[0207] For visualization of the bound biotinylated pdvWF the wells were incubated with o-Phenylenediamine dihydrochloride substrate (SIGMAFAST™ OPD, #P9187, Sigma Aldrich). Subsequently the absorbance at 492 nm was measured.

1.2 Results

[0208] As shown in Fig. 1 the absorbance at 492 nm increases with the starting concentration of vWF in the binding experiments with SIG-5, SIG-7, SIG-F, and SIG-9. The values are slightly above (SIG-F and SIG-9) or below (SIG-5 and SIG-7) the positive control (anti-vWF). In contrast, the absorbance in the binding experiments with SIG-2, SIG-10 and the negative control Anti-Chicken IgY was around 0 independent of the concentration.

[0209] Accordingly, vWF binds to SIG-5, SIG-7, SIG-F, and SIG-9 in a concentration-dependent manner. On the other hand, vWF does not bind to SIG-2 and SIG-10.

Example 2 - Binding of vWF fragments to SIGLECs

2.1 Experimental Procedure

[0210] C- and N-terminal fragments of vWF were prepared by V8 protease (Thermo Fisher Scientific, # 201959) digestion performed for 3 h, 37 °C, 300 rpm, using 1:100 enzyme to protein w/w ratio in a 50 mM Tris-HCl, 150 mM NaCl pH 7.8 buffer and purified by anion exchange chromatography on a MonoQ 5/50 GL column (GE Healthcare # 17-5166-01). The running buffer was 20 mM Tris-HCl pH 7.4, and the elution buffer 20 mM Tris-HCl, 500 mM NaCl pH 7.4. Fragments were further purified and desalted by size exclusion chromatography on a Superose 6 10/300 GL column (GE Healthcare # 17-5172-01) using 100mM NaCl as a running buffer.

[0211] The obtained fragments (C-terminal and N-terminal) are schematically depicted in Fig. 2 with an identification of domains and glycosylation sites.

[0212] The purified vWF C-terminal fragment and vWF N-terminal fragment were biotinylated using EZ-Link™ Sulfo-NHS-Biotin biotinylation kit (Thermo Fisher Scientific) and the binding to SIGLECs was measured as described in example 1 with a concentration of the vWF C-terminal fragment and vWF N-terminal fragment of 1 µg/mL.

2.2 Results

[0213] Based on the absorbance values shown in Fig. 3, the vWF N-terminal fragment, which contains the majority of the O-glycosylation sites and 2 O-glycan clusters (Cluster 1 and Cluster 2), binds to SIG-5, SIG-7, SIG-F and SIG-9. In contrast, little or no absorbance was measured for the vWF C-terminal fragment. Accordingly, the latter fragment does not bind to the SIGLECs.

Example 3 - Binding of N-terminal part of vWF to SIGLECs

3.1. Experimental Procedure

[0214] One portion of the N-terminal vWF fragment obtained in example 2 was enzymatically desialylated using Sialidase A . The incubation was performed at 37 °C for 3 h in 50 mM sodium phosphate, pH 6.0 using 2 µl enzyme for 100 µg VWF fragment (Sialidase A™ #GK80040 was obtained from Prozyme).

[0215] A second portion of the N-terminal vWF fragments was de-N-glycosylated. The incubation was performed over night at 37°C in 50 mM Sodium Phosphate pH 7.5 buffer using 1 µl enzyme for 20 µg VWF fragment (PNGase F #P0704 was obtained from New England Biolabs).

[0216] Samples of the desialylated, the de-N-glycosylated and the untreated N-terminal vWF fragment were tested for binding to SIG-5, SIG-7, SIG-8, and SIG-9. The binding experiment was carried out as described in Example 1 with a concentration of the N-terminal vWF fragments of 8 µg/mL.

3.2 Results:

[0217] As shown in Fig. 4, the absorbance values determined for the de-N-glycosylated vWF N-terminal fragment and the untreated vWF N-terminal fragment differs only slightly. Thus, de-N-glycosylation does not influence the binding, showing that the binding is mediated via O-glycans.

[0218] Desialylation of O-glycans strongly reduces or abolishes the binding of the vWF N-terminal fragment to SIGLECs as shown in Fig. 4. Thus, the binding of the vWF N-terminal fragment is mediated by the sialic acid attached to O-glycan chains.

Example 4 - SIGLEC binding of peptides containing O-glycan clusters 1 and 2

4.1 Experimental Procedure

[0219] vWF contains two clusters of fully occupied O-glycosylation sites (cf. Solecka et.al 2016), schematically depicted in Fig. 2. Both clusters differ in the relative amount of core 2 structures. Only 4.9% of the glycopeptide molecules contain core 2 structures in Cluster 1. Accordingly, the percentage of sialylated core 2 O-glycans based on the total number of O-glycans in cluster 1 is 1.25 %.

[0220] On the other hand, 34.86 % of the glycopeptide molecules contain core 2 structures in Cluster 2 (cf. Solecka et al., 2016). Accordingly, the percentage of sialylated core 2 O-glycans based on the total number of O-glycans in cluster 2 is 10.78%.

[0221] To measure the binding of the two clusters independently, the vWF N-terminal fragment was treated with Trypsin, producing the following fragment: Cluster 1 fragment encompassing AA 449 to 511 and Cluster 2 fragment encompassing AA 674 to 728/729 of VWF of SEQ ID NO: 1. The fragments were purified by reverse-phase HPLC. Briefly, pdVWF was reduced and

free cysteines were blocked with Maleimide-PEG2-Biotin according to manufacturer's instruction (EZ-Link™ Maleimide-PEG2-Biotin, #21901BID was obtained from Thermo Fisher Scientific). After digestion with trypsin at 37°C over night, high molecular weight peptides were concentrated using 10 kDa cut off centrifugal filter device (Millipore). Subsequently peptides were separated on a Jupiter 5µ 300Å C18 column (Phenomenex). The mobile phase was: A- 0.1% trifluoroacetic acid (TFA) in H₂O; B- 0.085% TFA in acetonitrile and the flow rate was 0.3 mL/min. Eluting peptides/glycopeptides were detected by ultraviolet absorption at 215 nm wavelength. Fractions of interest were collected, freeze-dried and subsequently reconstituted in 10 µL H₂O. Since both clusters contain cysteines, both were supplied with a biotin.

[0222] A binding experiment was carried out as described in Example 1 with the SIGLECs SIG-5, SIG-7, SIG-F, SIG-9 and SIG-10 and a concentration of the cluster 1 and cluster 2 fragments of 4 µg/mL.

[0223] Additionally, a sample of the cluster 1 and cluster 2 fragments were treated by desialylation and subsequently tested in a binding experiment as described in Example 1 with a concentration of the desialylated cluster 1 and cluster 2 fragments of 2 µg/mL.

4.2 Results:

[0224] The cluster 2 fragment bound to SIGLECs SIG-5, SIG-7, SIG-F, SIG-9 based on the absorbance values shown in Fig. 5. No or little absorbance was detected with SIG-10, confirming the results from the other examples.

[0225] Consequently, a high percentage of core 2 structures on O-glycan clusters is a requirement for binding to SIG-5, SIG-7, SIG-F, SIG-9.

Example 5 - Sialic acid dependency of Cluster 2 SIGLEC binding

5.1 Experimental Procedure

[0226] A sample of the cluster 2 fragment obtained as described in Example 4 was desialylated. The desialylated cluster 2 fragments and the untreated cluster 2 fragments were subsequently tested in a binding experiment as described in Example 1 with the SIGLECs SIG-5, SIG-7, SIG-F, and SIG-9 and a concentration of the desialylated Cluster 1 and Cluster 2 fragments of 2 µg/mL.

5.2 Results

[0227] The absorbance values detected for the untreated cluster 2 fragment confirmed the results found in Example 4 (see Fig. 6). De-sialylated cluster 2 fragment exhibits no or only little binding. Thus, sialylation of the core 2 structures on O-glycan clusters is a requirement for binding to SIG-5, SIG-7, SIG-F, and SIG-9.

Example 6 - Recombinant expression of VWF fragments with or without O-linked glycan repeats containing FVIII binding site

[0228] Two recombinant vWF fragments were expressed in the HEK cell line 293 F. The first fragment, Seq11 encompasses AA 1-505 of SEQ ID NO: 1 and contains Cluster 1 with the O-glycosylation sites 485, 492, 493, 500).

[0229] The second fragment Seq12 encompasses AA 1 to 505 of SEQ ID NO: 1 and 2 additional repetitions of the AAs 475-505 (AA 1-505 + 2x 475-505) and thus two additional copies of Cluster 1 O-glycan cluster repeats.

[0230] Seq11 and Seq12 were transiently expressed in the HEK293 cell line with a C-terminal Strep-Tag and purified by Strep-tactin affinity chromatography (IBA GmbH). Therefore, the cDNAs encoding Seq11 and Seq12, were synthesized by GeneArt (Thermo Fisher Scientific) and cloned in the pDSG- expression vector (IBA GmbH), containing a Twin-Strep-tag. TOP10 *E.coli* (IBA GmbH) were transformed with the constructs and single clones were selected following an overnight incubation at 37 °C on ampicillin-containing LB-agar plates. Plasmid DNA preparations were performed using the QIAamp DNA- Mini or Maxi kit (Qiagen) according to the manufacturer's recommendations. The correct orientation and integrity of the cloned constructs was verified by sequencing. For eukaryotic expression of both vWF fragments, MEXi-293 cells (IBA GmbH) grown in MEXi transfection-medium (IBA GmbH), were transfected with 1.5 mg/l of the constructs using 4.5 mg/ml 25 kDa linear polyethylenimine. After 2-4 hour incubation at 37 °C, 5 % CO₂ and 100-150 rpm, the culture was diluted 1:2 with MEXi transfection-medium and cultivation was continued until cell viability reached 75 %. Subsequently, the supernatant was separated from cells by centrifugation at 4°C and 300 x g. In order to minimize the inhibitory effect of biotin in the cell culture medium and to adjust the pH, 0.1 volumes of buffer (1M Tris-HCl, 1.5 mM NaCl, 10 mM EDTA, pH 8.0) and 0.09 % (v/v) BioLock solution (IBA GmbH) was added to the supernatant and incubated for 20 min at 4 °C. After centrifugation, the supernatant was applied on the Strep-Tactin XT column (IBA GmbH), washed five times with washing buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and bound Strep-tag containing proteins were eluted with elution buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM desthiobiotin, pH 8.0).

[0231] Both fragments Seq11 and Seq12 are schematically depicted in Fig. 7.

Example 7 - Analysis of the O-glycosylation of vWF fragments Seq11 and Seq12

7.1 Experimental Procedure

[0232] O-glycosylation of the fragments Seq11 and Seq12 produced according to Example 6 was analyzed by mass spectrometry.

[0233] For this, Seq11 and Seq12 were first reduced and alkylated by incubation with 50 mM dithiothreitol at 60°C and subsequently 20 min with 100 mM iodoacetamide. After digestion with trypsin and chymotrypsin, the obtained peptides were re-buffered in Sialidase A digestion buffer and desialylated over night using the conditions described in Example 3. The O-glycopeptides were specifically enriched by Jacalin (*Artocarpus integrifolia lectin*) affinity chromatography using agarose immobilized lectin (Vector Laboratories). Jacalin-agarose was packed into a gravity-driven column and the chromatography was performed according to the manufacturer's instructions. Eluted O-glycopeptides were purified for a MALDI MS measurement using C4 Ziptip pipette tips (Millipore) and subjected to a measurement in a linear positive ion mode using 25 mg/ml super DHB matrix dissolved in 50% Acetonitril/0.1% trifluoroacetic acid.

[0234] An aliquot of enriched glycopeptides was additionally treated with O-glycosidase (Endo- α -N-Acetylgalactosaminidase, #P0733, New England Biolabs). Briefly, peptides were incubated with the enzyme for 2 h at 37 °C using 1 μ l enzyme for 10 μ l glycoprotein. Since the O-glycosidase is specific for core 1 O-glycans only (Gal β 1 $\rightarrow$ 3GalNAc α 1 $\rightarrow$ Ser/Thr disaccharide), it leaves core 2 glycans and/or extended core 1 O-glycans attached to the peptide backbone.

7.2 Results

[0235] The results are summarized in Figures 8 and 9.

[0236] The O-glycopeptides were identified by a post source decay (PSD) MALDI. The peptide sequence of the identified Seq11 fragment is KVTLNPSDPEHCQICHCDWNL TCEACQEPGGL WPPTDAPVSPTTL YVEDISEP PLHGSAW (SEQ ID NO: 6). The last four amino acids (underlined) correspond to the C-terminal Strep-Tag. This peptide contains four O-glycosylation sites.

[0237] The upper spectrum of Fig. 8 shows the fully O-glycosylated glycopeptide, the lower spectrum of Fig. 8 shows the same glycopeptide after O-glycosidase digestion. The 1460.3 Da mass shift after O-glycosidase digestion (marked by an arrow), corresponds to four core 1 O-glycans each with a mass of 365 Da. 365 Da mass distances, observed in the upper spectrum, correspond to different glycoforms of the same peptide, whereas each additional 365 Da mass adduct corresponds to a Gal β 1 $\rightarrow$ 4GlcNAc disaccharide forming the core 2 structure. After O-glycosidase treatment, completely deglycosylated forms of the peptide (8358.6 Da) and

glycoforms containing core 2 structures (Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc, 730 Da) and/or extended core 1 structures (Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3GalNAc, 730 Da) are observed.

[0238] The identified peptide sequence of the Seq12 fragment is KVTLNPSDPEHCQICHCDWNL TCEACQEPGGL WPPTDAPVSPTTL YVEDISEP PLHQEPGGL WPPTDAPVSPTTL YVEDISEPPLHQEPGGL WPPTDAPVSPTTL Y VEDISEPPLH GSAW (SEQ ID NO: 7). The last four amino acids (underlined) correspond to the C-terminal Strep-Tag.

[0239] The upper spectrum of Fig. 9 shows the fully O-glycosylated glycopeptide, the lower spectrum of Fig. 9 shows the same glycopeptide after O-glycosidase digestion. The 4380.3 Da mass shift after O-glycosidase digestion, corresponds to twelve core 1 O-glycans each with a mass of 365 Da, which confirms that all twelve O-glycosylation sites in Seq12 are occupied by O-glycans. Similarly, as observed for Seq11, 365 Da and 730 Da distances observed in the spectra correspond to the Gal β 1 $\rightarrow$ 4GlcNAc disaccharide or to core 2 and/or extended core 1 structures, respectively.

[0240] Quantitation of core 1 and core 2 type O-glycans is based on the relative quantity of the glycopeptides with the respective O-glycan attached. The quantitation of the different glycoforms of a given peptide is done by evaluation of the signal intensity in the MALDI spectrum.

[0241] For the glycopeptide Seq11, the quantitation is done based on the MALDI signal intensity of the different glycoforms of the same peptide. The total peak intensity of all glycoforms of this peptide (8358 Da, 8724 Da, 9089 Da, 9454 Da, 9819 Da, 10181 Da, 10543 Da, 10910 Da, 11277 Da) equals 41275 a.u., which represents 100%.

[0242] The glycoform containing only core 1 glycans with a mass 8358 Da exhibits an intensity of 8410 a.u., which corresponds to 20% of the total. Thus, all other glycoforms (80%) contain at least one core 2 and/or extended core 1 glycan type glycan attached. In this measurement core 2 and extended core 1 are not distinguishable. Accordingly, the percentage of core 2 and/or extended core 1 O-glycans based on the total number of O-glycans is at least 20 %.

[0243] Accordingly, the four O-glycosylation sites in Seq11 and all twelve O-glycosylation sites in Seq12, both recombinantly produced in a HEK cell line, are occupied with core 1 O-glycans and to a high percentage also with core 2 O-glycans and/or extended core 1 O-glycans. Considering the high amount of core 2 O-glycans both sequences can be good ligands for SIGLEC binding. The addition of two additional sequence repeats, containing four O-glycosylation sites, successfully resulted in a protein with twelve clustered and fully occupied O-glycans.

Example 8 - Analysis SIGLEC binding of Seq11 and Seq12

8.1 Experimental Procedure

[0244] Strep-Tag bearing recombinant proteins Seq11 and Seq12 were tested in the SIGLEC binding ELISA as described in Example 1, except for the detection strategy. Instead of the Streptavidin-HRP, Strep-Tactin-HRP (#2-1502-001, IBA GmbH) conjugate was used for detection of the Strep-tagged proteins. The concentration of the applied Strep-Tactin-HRP was 0.25 µg/ml. Both proteins, Seq11 and Seq12 were tested in equal molar concentration of 42 nM.

8.2 Results

[0245] As shown in Fig. 10, both polypeptides Seq11 and Seq12 exhibited a binding to SIG-5, SIG-7, SIG-F and SIG-9. The measured absorbance for both polypeptides Seq11 and Seq12 to all four SIGLECs was in the same range as the binding of Seq11 and Seq12 to anti-vWF.

[0246] In contrast, the absorbance of Seq11 and Seq12 in the experiment with SIG-2 and SIG10 was in the same range as the negative control experiment with anti-chicken antibody. Thus, neither did Seq11 nor Seq12 bind to SIG-2 or to SIG-10 (see Fig. 10).

Example 9 - Sialic Acid dependency of SIGLEC binding of Seq11 and Seq12

9.1 Experimental Procedure

[0247] The experimental protocol according to Example 8 was repeated with the addition of a Sialidase A digestion of the strep-tagged polypeptides Seq11 and Seq12. The desialylation was carried out as described in Example 3.

9.2 Results

[0248] The results of the binding experiments with desialylated Seq11 and Seq12 are shown in Fig. 11. According to the measured absorbances, the binding of desialylated Seq11 and Seq12 to SIG-5 is strongly reduced in comparison to the untreated polypeptides. The binding to SIG-7, SIG-F and SIG-9 is completely abolished, i.e. the absorbance is at the same level as determined for binding to SIG-2 and SIG10. Thus, binding of both polypeptides Seq11 and Seq12 to SIG-5, SIG-7, SIG-F and SIG-9 is sialic acid dependent.

Example 10 - Comparison of SIGLEC binding of Seq11 and Seq12

10.1 Experimental Procedure

[0249] In order to measure and compare the apparent binding affinities of Seq11 and Seq12, SIGLEC ELISA with Scatchard analysis of the binding curves was applied. The ELISA was performed as described in Examples 8 and 9. Scatchard analysis was done using Graph Pad Prism software.

10.2 Results

[0250] The binding curves of the Sequences 11 and 12 and the corresponding Scatchard plots are depicted in figures 12 and 13. The apparent binding affinities (K_D) derived from the Scatchard plots are summarized in Fig. 14. The increase in O-glycan repeats in Seq12 had a significant effect on SIGLEC binding affinity. The affinity for SIG-5 could be increased from 0.494 μ M for Seq11 to 0.14 μ M for Seq12. The affinity for SIG-7 could be increased from 0.371 μ M for Seq11 to 0.005 μ M for Seq12. The affinity for SIG-8 could be increased from 1.027 μ M for Seq11 to 0.015 μ M for Seq12. Finally, the affinity for SIG-9 could be increased from 0.591 μ M for Seq11 to 0.041 μ M for Seq12.

Example 11 - Determination of the FVIII binding affinity of Seq11 and Seq12

11.1 Experimental Procedure

[0251] FVIII binding of both sequences was evaluated by surface plasmon resonance (SPR). The analysis was carried out using a Biacore 3000 (GE Healthcare) instrument. The Sequence 11 and 12 polypeptides were immobilized on a CM5 chip using an amine coupling kit (GE Healthcare). As a positive control full-length plasmatic VWF (Wilate, Octapharma) was immobilized. Subsequently, a series of FVIII (Nuwiq, Octapharma) concentrations (0.2 nM, 0.6 nM, 1.7 nM, 5.0 nM, 15 nM, 45 nM) was injected over the sensor chip surface. The running buffer was 150 mM HEPES, 150 mM NaCl, 5 mM CaCl_2 , 0.05 %.

11.2 Results

[0252] SPR measurement revealed that FVIII binding affinity (K_D) of both sequences equals 1.4 nM. The additional O-glycan repeats have therefore no impact on the binding affinity to FVIII.

Example 12 - The N-terminal, but not the C-terminal VWF fragment reduces the extracellular level of IL-12p70 and IFN- γ

12.1 Background

[0253] SIGLECs are expressed on various cells of the immune system, including monocytes and dendritic cells, and exhibit a role in cell adhesion and endocytosis. They also modulate signaling pathways of the adaptive and innate immunity (Macauley et al., 2014). Most SIGLECs contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) or ITIM-like motif in their cytoplasmic domain, which has been shown to function in attenuation of the inflammatory response by inhibiting cellular proliferation and activation (Vitale et al., 1999; Ikehara et al., 2004), inducing apoptosis (Nutku et al., 2003) and suppressing cytokine production (Erdmann et al. 2009; Chen et al., 2013).

[0254] In order to see if the levels of inflammatory cytokines produced by immature monocyte derived dendritic cells (moDCs) are altered in the presence of the SIGLEC-engaging vWF-fragments, amounts of IL-12p70 and IFN- γ in the supernatant of stimulated moDCs were analyzed simultaneously by flow cytometry.

12.2 Experimental design

[0255] Monocytes from healthy donors were enriched via Ficoll gradient and subsequently CD14⁺ monocytes were purified by magnetic cell sorting. To obtain moDCs, CD14⁺ monocytes were cultivated for 5 to 6 days in RPMI medium supplemented with 10 % fetal calf serum, 1000 U/ml interleukin 4 and 1000 U/ml granulocyte-macrophage colony-stimulating factor. The cytokine profile secreted by the moDCs was analyzed 24 h post stimulation with the respective vWF fragments via the cytometric bead array CBA Flex (BD) detecting IL-12p70 and IFN- γ according to the manufacturer's recommendation. Cells treated with the same volume of 100 mM NaCl served as control. Samples were analyzed with the flow cytometer FACSVerse. Final analysis and calculation of the cytokine concentration was carried out using FCAP Array software (BD).

12.3 Results

[0256] Figure 16 shows the cytokine concentration after incubation of the moDCs with the two vWF fragments with and without LPS stimulation. According to these results, the N-terminal, but not the C-terminal part of vWF lowers the production of pro-inflammatory cytokines synthesized in response to LPS stimulation. Without LPS stimulation, no effect of the vWF

fragments on secretion of the pro-inflammatory cytokines could be detected.

Example 13 - Analysis of phosphorylation of SIGLECs and their adaptor molecules

13.1 Background

[0257] Upon sialic acid-containing ligand binding, the ITIM and ITIM-like motifs of SIGLECs become phosphorylated by SRC-family tyrosine kinases, which leads to the recruitment of SRC homology 2 (SH2)-domain containing protein tyrosine phosphatases (SHP)-1 and SHP-2. Once activated, these phosphatases can dephosphorylate cellular substrates, thereby controlling the activation of various signaling pathways (Crocker et al., 2007). While SHP-1 has been ascribed a role in inhibitory signaling, SHP-2 enhances signal transduction in most signaling pathways but has also been reported to be involved in negatively regulating intracellular signaling processes (An et al., 2006; Avril et al., 2004; Boyd et al., 2009; Qu, 2000; Salmond and Alexander, 2006).

13.2 Experimental Procedure

[0258] MoDCs were prepared as described in example 12. In order to determine the influence of the N-terminal vWF-fragment on tyrosine phosphorylation of SIGLECs and their adaptor molecules SHP-1 and SHP-2, 6×10^6 moDCs were incubated for 10 minutes with 500 nM of the N-terminal vWF fragment. Cells stimulated with the same volume of 100 mM NaCl served as a control. Analysis of immunoreceptor-phosphorylation was carried out with the Proteome Profiler Human Phospho-Immunoreceptor Array Kit (R&D systems) according to the manufacturer's recommendations using 500 µg cell lysate.

13.3 Results

[0259] The results of the Phospho-Immunoreceptor Array are shown in Fig. 17. According to the measured pixel density, the N-terminal vWF fragment specifically alters the phosphorylation of SHP-1, SHP-2, SIG-5 and SIG-7 as compared to control. The phosphorylation of SIG-2 and SIG-10 could not be observed, which is in close accordance with the lack of binding of the vWF N-terminal fragment to these SIGLECs.

REFERENCES

[0260]

An,H., Zhao,W., Hou,J., Zhang,Y., Xie,Y., Zheng,Y., Xu,H., Qian,C., Zhou,J., Yu,Y., Liu,S., Feng,G., and Cao,X. (2006). SHP-2 phosphatase negatively regulates the TRIF adaptor protein-dependent type I interferon and proinflammatory cytokine production. *Immunity*. 25, 919-928.

Avril,T., Floyd,H., Lopez,F., Vivier,E., and Crocker,P.R. (2004). The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. *J. Immunol*. 173, 6841-6849.

Boyd.C.R., Orr,S.J., Spence,S., Burrows, J. F., Elliott,J., Carroll,H.P., Brennan,K., Ni,G.J., Coulter,W.A., Jones,C., Crocker,P.R., Johnston,J.A., and Jefferies,C.A. (2009). Siglec-E is up-regulated and phosphorylated following lipopolysaccharide stimulation in order to limit TLR-driven cytokine production. *J. Immunol*. 183, 7703-7709.

Canis K, McKinnon TA, Nowak A, Haslam SM, Panico M, Morris HR, Laffan MA, Dell A. (2012) Mapping the N-glycome of human von Willebrand factor. *Biochem J*. Oct 15;447(2):217-28.

Chen, Weilin; Han, Chaofeng; Xie, Bin; Hu, Xiang; Yu, Qian; Shi, Liyun et al. (2013): Induction of Siglec-G by RNA viruses inhibits the innate immune response by promoting RIG-I degradation. In: *Cell* 152 (3), S. 467-478. DOI: 10.1016/j.cell.2013.01.011.

Crocker,P.R., Paulson,J.C., and Varki,A. (2007). Siglecs and their roles in the immune system. *Nat. Rev. Immunol*. 7, 255-266.

Ewenstein BM, Collins P, Tarantino MD, Negrier C, Blanchette V, Shapiro AD, Baker D, Spotts G, Sensel M, Yi SE, Gomperts ED. Hemophilia therapy innovation development of an advanced category recombinant factor VIII by a plasma/albumin-free method Proceedings of a Special Symposium at the XIXth Congress of the International Society on Thrombosis and Haemostasis; 2004, vol. 41, pg. 1-16.

Erdmann, Hanna; Steeg, Christiane; Koch-Nolte, Friedrich; Fleischer, Bernhard; Jacobs, Thomas (2009): Sialylated ligands on pathogenic *Trypanosoma cruzi* interact with Siglec-E (sialic acid-binding Ig-like lectin-E). In: *Cellular microbiology* 11 (11), S. 1600-1611. DOI: 10.1111/j.1462-5822.2009.01350.x.

Franc V, Řehulka P, Raus M, Stulik J, Novak J, Renfrow MB, Šebela M. Elucidating heterogeneity of IgA1 hinge-region O-glycosylation by use of MALDI-TOF/TOF mass spectrometry: role of cysteine alkylation during sample processing. *Journal of Proteomics*, 2013, Oct 30; vol. 92, pg. 299-312

Guzman-Aranguez A, Argüeso P. Structure and Biological Roles of Mucin-type O-glycans at the Ocular Surface. *The ocular surface*. 2010; vol. 8 (1) pg- 8-17.

Ikehara, Yuzuru; Ikehara, Sanae Kabata; Paulson, James C. (2004): Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. In: *The Journal of biological chemistry* 279 (41), S. 43117-43125. DOI: 10.1074/jbc.M403538200.

Lai JD Georgescu MT, Hough C, Lillicrap D. To clear or to fear: An innate perspective on factor VIII immunity, *Cellular Immunology*, 2016 Mar; vol. 301, pg. 82-89.

Lenting PJ, Casari C, Christophe OD, Denis CV. von Willebrand factor: the old, the new and the unknown. *J Thromb Haemost.* 2012 Dec;10(12):2428-37

Nutku, Esra; Aizawa, Hideyuki; Hudson, Sherry A.; Bochner, Bruce S. (2003): Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. In: *Blood* 101 (12), S. 5014-5020. DOI: 10.1182/blood-2002-10-3058.

Pegon JN, Kurdi M, Casari C, Odouard S, Denis CV, Christophe OD, Lenting PJ. Factor VIII and von Willebrand factor are ligands for the carbohydrate-receptor Siglec-5, *Haematologica*. 2012 Dec; 97(12):1855-63.

Paulson JC, Macauley MS, and Kawasaki N. Siglecs as sensors of self in innate and adaptive immune responses. *Ann N Y Acad Sci.* 2012 April; 1253(1): 37-48.

Qu,C.K. (2000). The SHP-2 tyrosine phosphatase: signaling mechanisms and biological functions. *Cell Res.* 10, 279-288.

Salmond,R.J., and Alexander,D.R. (2006). SHP2 forecast for the immune system: fog gradually clearing. *Trends Immunol.* 27, 154-160.

Solecka BA, Weise C, Laffan MA, Kannicht C, Site-specific analysis of von Willebrand factor O-glycosylation, *J Thromb Haemost.* 2016 Jan 19.

Vitale, C.; Romagnani, C.; Falco, M.; Ponte, M.; Vitale, M.; Moretta, A. et al. (1999): Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells. In: *Proceedings of the National Academy of Sciences of the United States of America* 96 (26), S. 15091-15096.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- [WO2014176125A1](#) [0008]
- [WO2014179184A1](#) [0010]
- [WO2013120939A1](#) [0015]
- [WO2015185758A2](#) [0058] [0058]

Non-patent literature cited in the description

- **SAENKO et al.** Haemophilia, 2002, vol. 8, 1-11 [0005]
- **NEEDLEMANWUNSCHJ.** Mol. Biol., 1970, vol. 48, 443-453 [0037]
- **RICEEMBOSS:** The European Molecular Biology Open Software Suite Trends Genet., 2000, vol. 16, 276-277 [0037]
- **INNIS et al.** PCR: A Guide to Methods and Application Academic Press 1990 0000 [0181]
- **GOODMANGILMAN'S et al.** The Pharmacological Basis of Therapeutics McGraw-Hill Professional 2001 0000 [0200]
- Remington: The Science and Practice of Pharmacy Lippincott Williams & Wilkins 2003 0000 [0200]
- Pharmaceutical Dosage Forms and Drug Delivery Systems Lippincott Williams & Wilkins 1999 0000 [0200]
- **AN, H. ZHAO, W. HOU, J. ZHANG, Y. XIE, Y. ZHENG, Y. XU, H. QIAN, C. ZHOU, J. YU, Y. SHP-2** phosphatase negatively regulates the TRIF adaptor protein-dependent type I interferon and proinflammatory cytokine production Immunity, 2006, vol. 25, 919-928 [0260]
- **AVRIL, T. FLOYD, H. LOPEZ, F. VIVIER, E. CROCKER, P. R.** The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells J. Immunol., 2004, vol. 173, 6841-6849 [0260]
- **BOYD, C. R. ORR, S. J. SPENCE, S. BURROWS, J. F. ELLIOTT, J. CARROLL, H. P. BRENNAN, K. NI, G. J. COULTER, W. A. JONES, C.** Siglec-E is up-regulated and phosphorylated following lipopolysaccharide stimulation in order to limit TLR-driven cytokine production J. Immunol., 2009, vol. 183, 7703-7709 [0260]
- **CANIS KMCKINNON TANOWAK AHASLAM SMPANICO MMORRIS HRLAFFAN MADELL A.** Mapping the N-glycome of human von Willebrand factor Biochem J., 2012, vol. 447, 2217-28 [0260]
- **CHEN, WEILINHAN, CHAOFENGXIE, BINHU, XIANGYU, QIANSHI, LIYUN et al.** Induction of Siglec-G by RNA viruses inhibits the innate immune response by promoting RIG-I degradation Cell, 2013, vol. 152, 3467-478 [0260]
- **CROCKER, P. R. PAULSON, J. C. VARKI, A.** Siglecs and their roles in the immune system Nat. Rev. Immunol., 2007, vol. 7, 255-266 [0260]
- **EWENSTEIN BMCOLLINS PTARANTINO MDNEGRIER CBLANCHETTE VSHAPIRO**

ADBAKER DSPOTTS GSENSEL MYI SEHemophilia therapy innovation development of an advanced category recombinant factor VIII by a plasma/albumin-free methodProceedings of a Special Symposium at the XIXth Congress of the International Society on Thrombosis and Haemostasis, 2004, vol. 41, 1-16 [0260]

- **ERDMANN, HANNASTEEG, CHRISTIANEKOCH-NOLTE, FRIEDRICHFLEISCHER, BERNHARDACOBS, THOMAS**Sialylated ligands on pathogenic Trypanosoma cruzi interact with Siglec-E (sialic acid-binding Ig-like lectin-E)Cellular microbiology, 2009, vol. 11, 111600-1611 [0260]
- **GUZMAN-ARANGUEZ AARGÜESO P**Structure and Biological Roles of Mucin-type O-glycans at the Ocular SurfaceThe ocular surface., 2010, vol. 8, 18-17 [0260]
- **IKEHARA, YUZURUIKEHARA, SANAE KABATAPaulson, James C.**Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9The Journal of biological chemistry, 2004, vol. 279, 4143117-43125 [0260]
- **LAI JD GEORGESCU M**THOUGH CLILLICRAP DTo clear or to fear: An innate perspective on factor VIII immunityCellular Immunology, 2016, vol. 301, 82-89 [0260]
- **LENTING PJCASARI CCHRISTOPHE ODDENIS CV**von Willebrand factor: the old, the new and the unknownJ Thromb Haemost, 2012, vol. 10, 122428-37 [0260]
- **NUTKU, ESRAAIZAWA, HIDEYUKIHudson, Sherry A.Bochner, Bruce S.**Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosisBlood, 2003, vol. 101, 125014-5020 [0260]
- **PEGON JNKURDI MCASARI CODOUARD SDENIS CVCHRISTOPHE ODLENTING PJ**Factor VIII and von Willebrand factor are ligands for the carbohydrate-receptor Siglec-5Haematologica, 2012, vol. 97, 121855-63 [0260]
- **PAULSON JCMACAULEY MSKAWASAKI N**Siglecs as sensors of self in innate and adaptive immune responsesAnn N Y Acad Sci., 2012, vol. 1253, 137-48 [0260]
- **QU,C.K.**The SHP-2 tyrosine phosphatase: signaling mechanisms and biological functionsCell Res., 2000, vol. 10, 279-288 [0260]
- **SALMOND,R.J.ALEXANDER,D.R.**SHP2 forecast for the immune system: fog gradually clearingTrends Immunol., 2006, vol. 27, 154-160 [0260]
- **SOLECKA BAWEISE CLAFFAN MAKANNICHT C**Site-specific analysis of von Willebrand factor O-glycosylationJ Thromb Haemost., 2016, [0260]
- **VITALE, C.ROMAGNANI, C.FALCO, M.PONTE, M.VITALE, M.MORETTA, A. et al.**Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cellsProceedings of the National Academy of Sciences of the United States of America, 1999, vol. 96, 2615091-15096 [0260]

POLYPEPTIDMODULERENDE SIGLEC-AFHÆNGIGE IMMUNREAKTIONER

PATENTKRAV

- 5 1. Glykosyleret polypeptid omfattende en aminosyresekvens, der er mindst 90 % identisk med mindst et fragment af human vWF (SEQ ID NO: 1), hvor det glykosylerede polypeptid indeholder et eller flere clustre af O-glykosyleringssteder, hvor clustrene indeholder mindst to O-glykosyleringssteder og et eller flere sialylerede O-glykaner, hvor det kombinerede antal sialylerede core 2- og udvidede core 1-O-glykaner af det glykosylerede polypeptid er større end
10 antallet af sialylerede core 2- og udvidede core 1-O-glykaner af den humane vWF eller fragmentet deraf, og hvor det glykosylerede polypeptid viser øget bindingsaffinitet til et eller flere SIGLEC'er, valgt fra gruppen bestående af SIG-5, SIG-7, SIG-8 og SIG-9 sammenlignet med den humane vWF eller fragmentet deraf.
- 15 2. Glykosyleret polypeptid ifølge krav 1, hvor antallet af sialylerede core 2-O-glykaner af det glykosylerede polypeptid er større end antallet af sialylerede core 2-O-glykaner af det humane protein eller fragmentet deraf.
- 20 3. Glykosyleret polypeptid ifølge et hvilket som helst af de foregående krav, hvor det ene eller flere clustre indeholder mindst tre O-glykosyleringssteder og fortrinsvist mindst fire O-glykosyleringssteder, og/eller det ene eller flere clustre indeholder mindst et O-glykosyleringssted i ti aminosyrer, fortrinsvist mindst et O-glykosyleringssted i fire aminosyrer, mere fortrinsvist mindst et O-glykosyleringssted i tre aminosyrer og mest fortrinsvist et O-glykosyleringssted i to aminosyrer.
- 25 4. Glykosyleret polypeptid ifølge et hvilket som helst af krav 1 til 3, indeholdende mindst to clustre, mere fortrinsvist mindst tre clustre, hvor clustrene fortrinsvist er adskilt af færre end 100 aminosyrer, mere fortrinsvist af færre end 50, mest fortrinsvist af færre end 30 aminosyrer.
- 30 5. Glykosyleret polypeptid ifølge et hvilket som helst af de foregående krav, hvor andelen af sialyleret core 2-O-glykaner baseret på antallet af sialylerede O-glykaner er fortrinsvist mindst 5 %, mere fortrinsvist mindst 10 %, mest fortrinsvist mindst 35 %.

6. Glykosyleret polypeptid ifølge et hvilket som helst af de foregående krav, hvor det glykosylerede polypeptids samlede antal O-glykosyleringssteder er større end det samlede antal O-glykosyleringssteder for den humane vWF eller fragmentet deraf.

5

7. Glykosyleret polypeptid ifølge krav 6, hvor et eller flere O-glykosyleringssteder befinder sig inden for aminosyresekvensen, der er homolog eller identisk med det humane protein eller fragmentet deraf, især erstattende aminosyrer i det humane protein eller fragmentet deraf.

- 10 8. Glykosyleret polypeptid ifølge krav 6, hvor det glykosylerede polypeptid er et fusionsprotein, hvor en anden aminosyresekvens indeholdende et eller flere O-glykosylationssteder er kovalent linket til aminosyresekvensen, der er identisk eller homolog med det humane protein eller fragmentet deraf, hvor den kovalente linker fortrinsvist er valgt fra gruppen bestående af en peptinbinding, en kemisk linker og en glycosidbinding.

15

9. Glykosyleret polypeptid ifølge krav 8, hvor den anden aminosyresekvens er mindst 98 % homolog, fortrinsvist identisk, med aminosyrer 475 til 505 i SEQ ID NO: 1 eller med to på hinanden følgende kopier af aminosyrer 475 til 505 i SEQ ID NO: 1.

- 20 10. Glykosyleret polypeptid ifølge et hvilket som helst af krav 1 til 9 produceret ved hjælp af ekspresion i en human cellelinje.

11. Anvendelse af et glykosyleret polypeptid ifølge et hvilket som helst af krav 1-10, indeholdende et eller flere yderligere clustre af sialylerede O-glykaner og udvisende øget binding til en eller flere SIGLEC'er, valgt fra gruppen bestående af SIG-5, SIG-7, SIG-8 og SIG-9 til reduktion af et andet polypeptids, især et terapeutisk proteins, immunreaktion.

25

12. Sammensætning omfattende et første og et andet polypeptid, hvor det første polypeptid er et glykosyleret polypeptid ifølge et hvilket som helst af krav 1 til 10, og det andet polypeptid indeholder en aminosyresekvens, der er homolog eller identisk med et andet pattedyrsprotein, især et humant protein, hvor det første og det andet polypeptid danner et proteinkompleks, og hvor sammensætningen, sammenlignet med det andet polypeptid, har øget bindingsaffinitet til et

30

SIGLEC valgt blandt et eller flere SIGLEC'er, valgt fra gruppen bestående af SIG-5, SIG-7, SIG-8 og SIG-9.

13. Sammensætning ifølge krav 12, hvor det andet polypeptid er valgt fra gruppen bestående af et
5 FVIII-protein, FVII, FIX, ADAMTS13, især et FVIII-protein i fuld længde, et B-domæne-deleteret FVIII-protein eller et FVIII-protein, i hvilket en del af B-domænet er blevet erstattet af en linker.
14. Sammensætning ifølge krav 11 eller 12, hvor det første polypeptid omfatter mindst et fragment af
10 human vWF, især vWF-fragmentet er mindst 95 % identisk med sekvensen defineret af 1 til 505 af SEQ ID NO: 1, mindst 95 % identisk med SEQ ID NO: 2, mindst 95 % identisk med SEQ ID NO: 3, mindst 95 % identisk med SEQ ID NO: 4 eller mindst 95 % identisk med SEQ ID NO: 5, og det andet polypeptid er et FVIII-protein.
15. Isoleret polynukleotid, der koder for et glykosyleret polypeptid ifølge et hvilket som helst af krav
15 6 til 9.
16. Vektor indeholdende et vektor-backbone og polynukleotidet ifølge krav 15, hvor vektor-
backbonet fortrinsvist er valgt fra gruppen bestående af pCDNA3, pCDNA3.1, pCDNA4, pCDNA5, pCDNA6, pCEP4, PCEP-puro, pCET1019, pCMV, pEF1, pEF4, pEF5, pEF6,
20 pExchange, pEXPR, pIRES og pSCAS.
17. Værtscelle indeholdende polynukleotidet ifølge krav 15 eller vektoren ifølge krav 16, hvor
værtscellen fortrinsvist er en pattedyrscelle, fortrinsvist en human celle, mere fortrinsvist en human nyrecelle, mest fortrinsvist en human embryonisk nyrecellelinje, især en HEK293-
25 cellelinje såsom HEK293F.
18. Glykosyleret polypeptid defineret ifølge et hvilket som helst af krav 1 til 10 eller sammensætning
defineret ifølge et hvilket som helst af krav 12 til 14, til anvendelse i behandlingen eller forebyggelsen af en hæmoragisk sygdom, fortrinsvist valgt blandt behandling af tidligere
30 ubehandlede patienter (PUP'er) og immuntoleranceinduktions- (ITI)-behandling.

DRAWINGS

Drawing

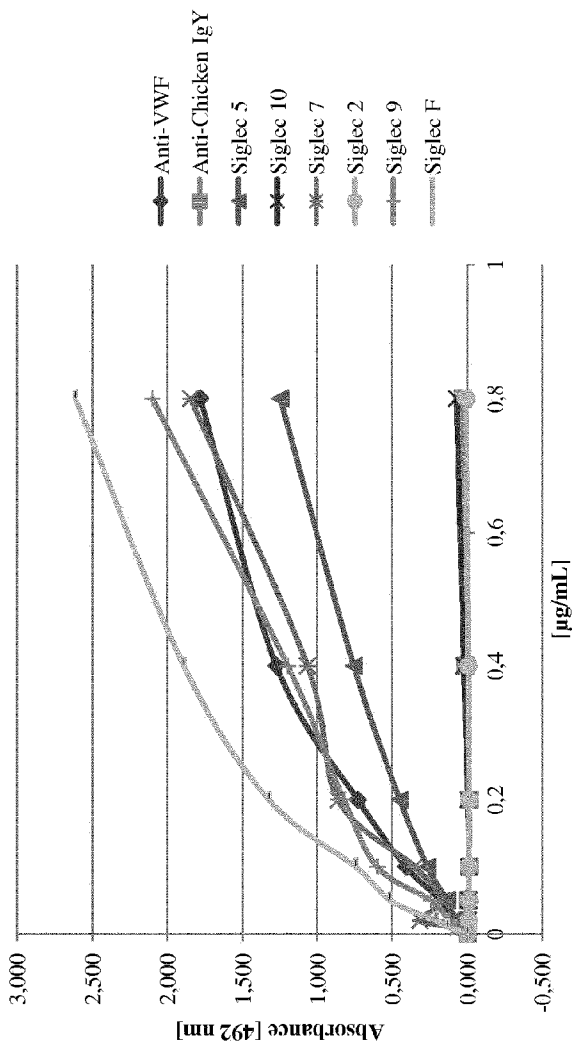


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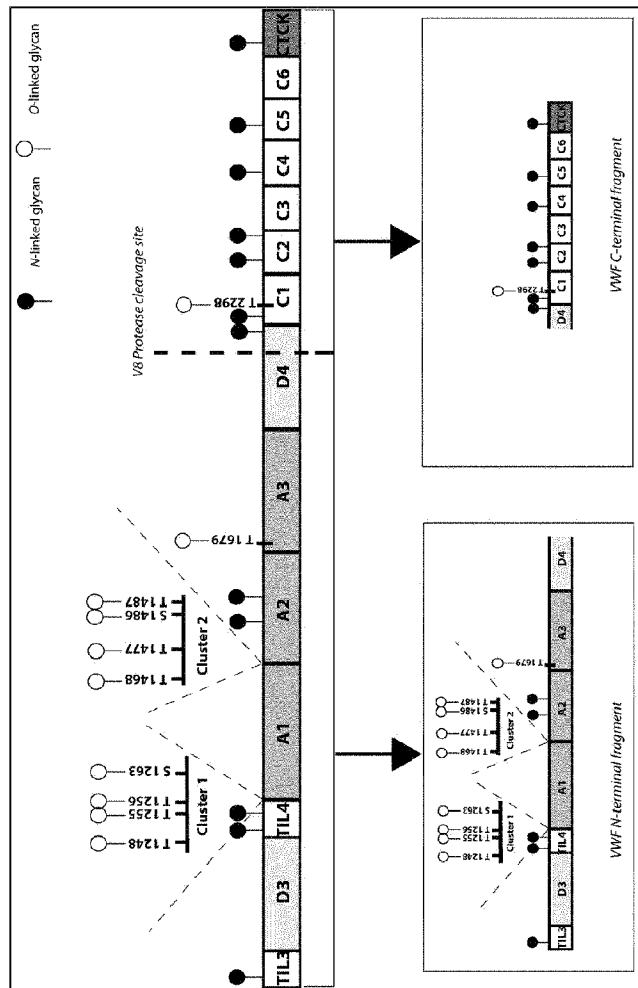
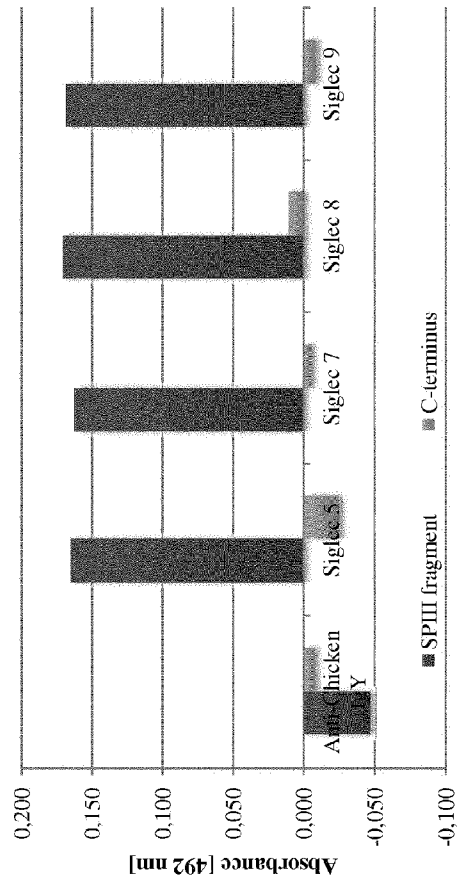
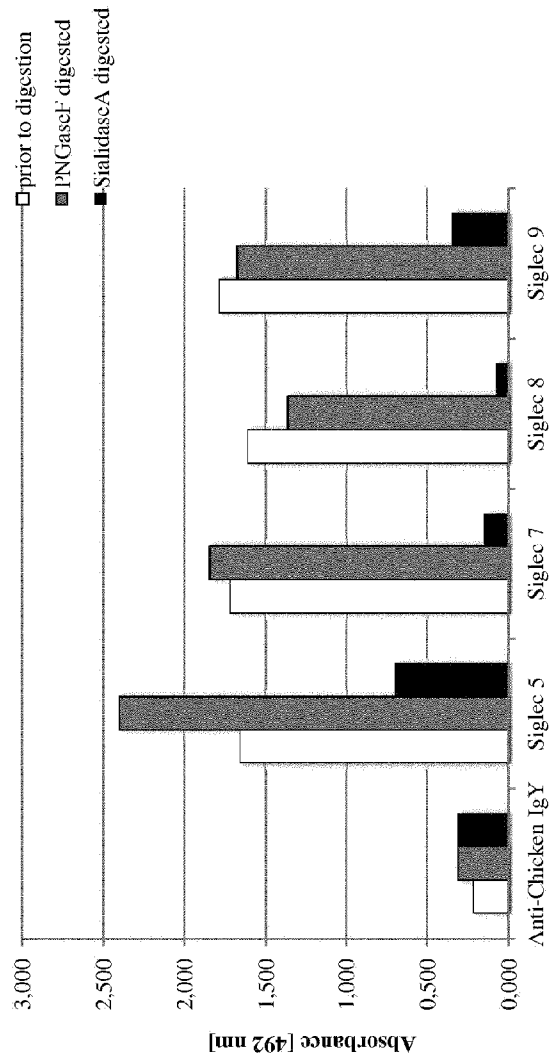
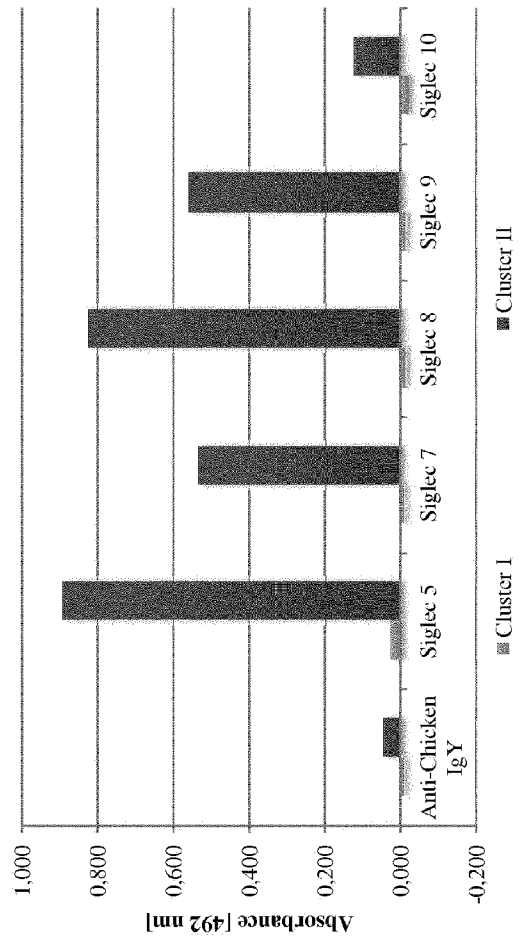
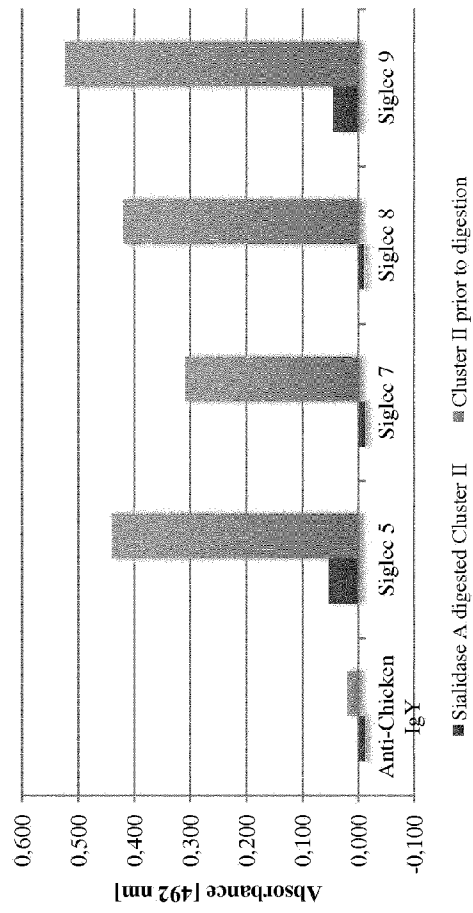


Fig. 2

**Fig. 3**

**Fig. 4**

**Fig. 5**

**Fig. 6**

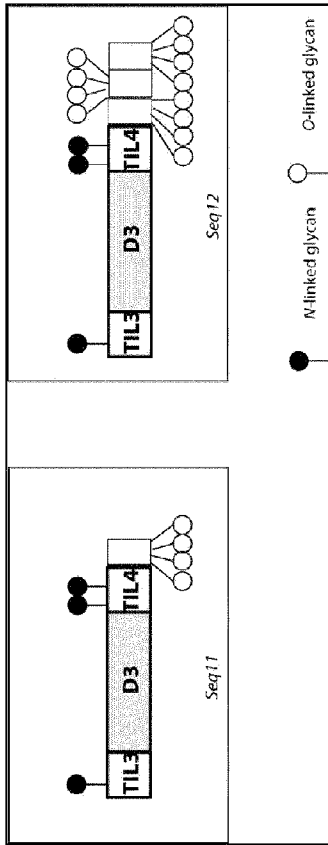


Fig. 7

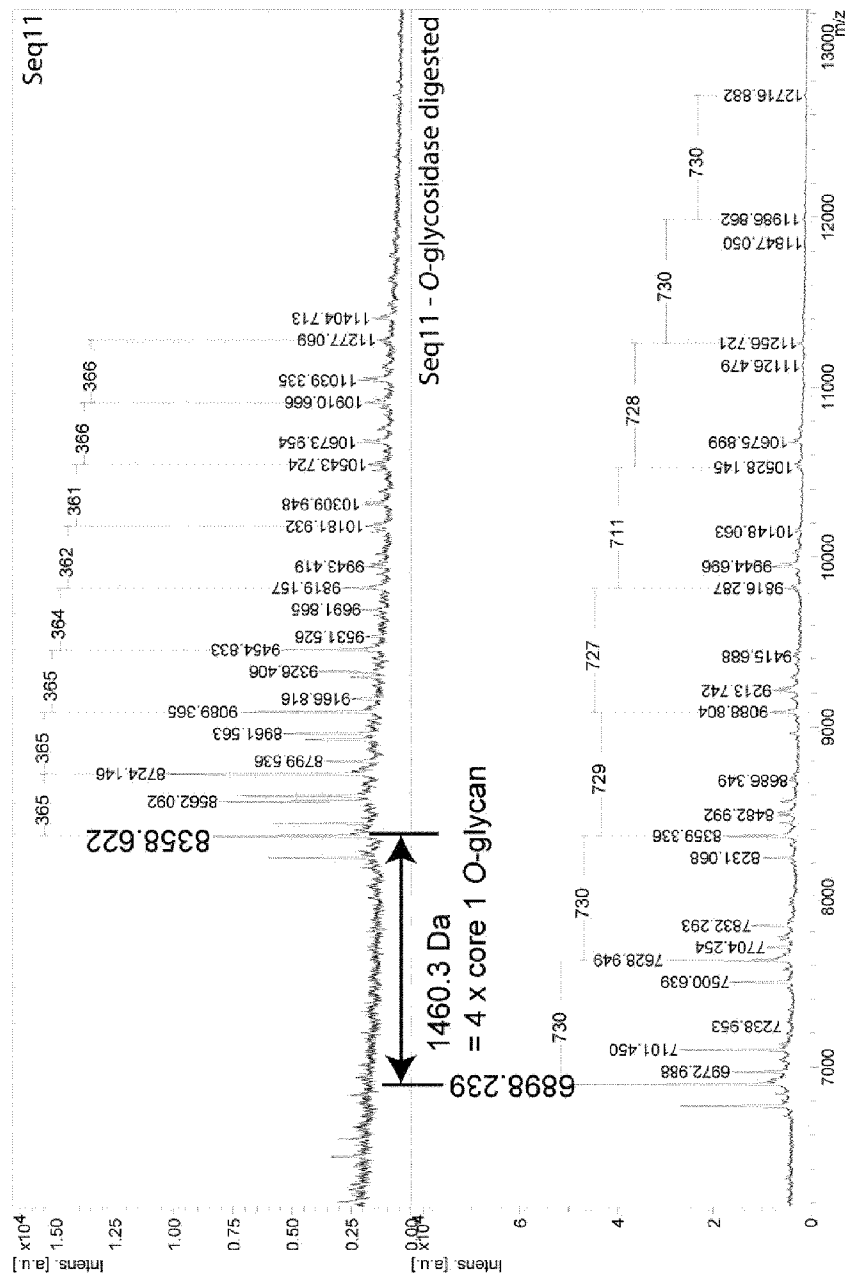


Fig. 8

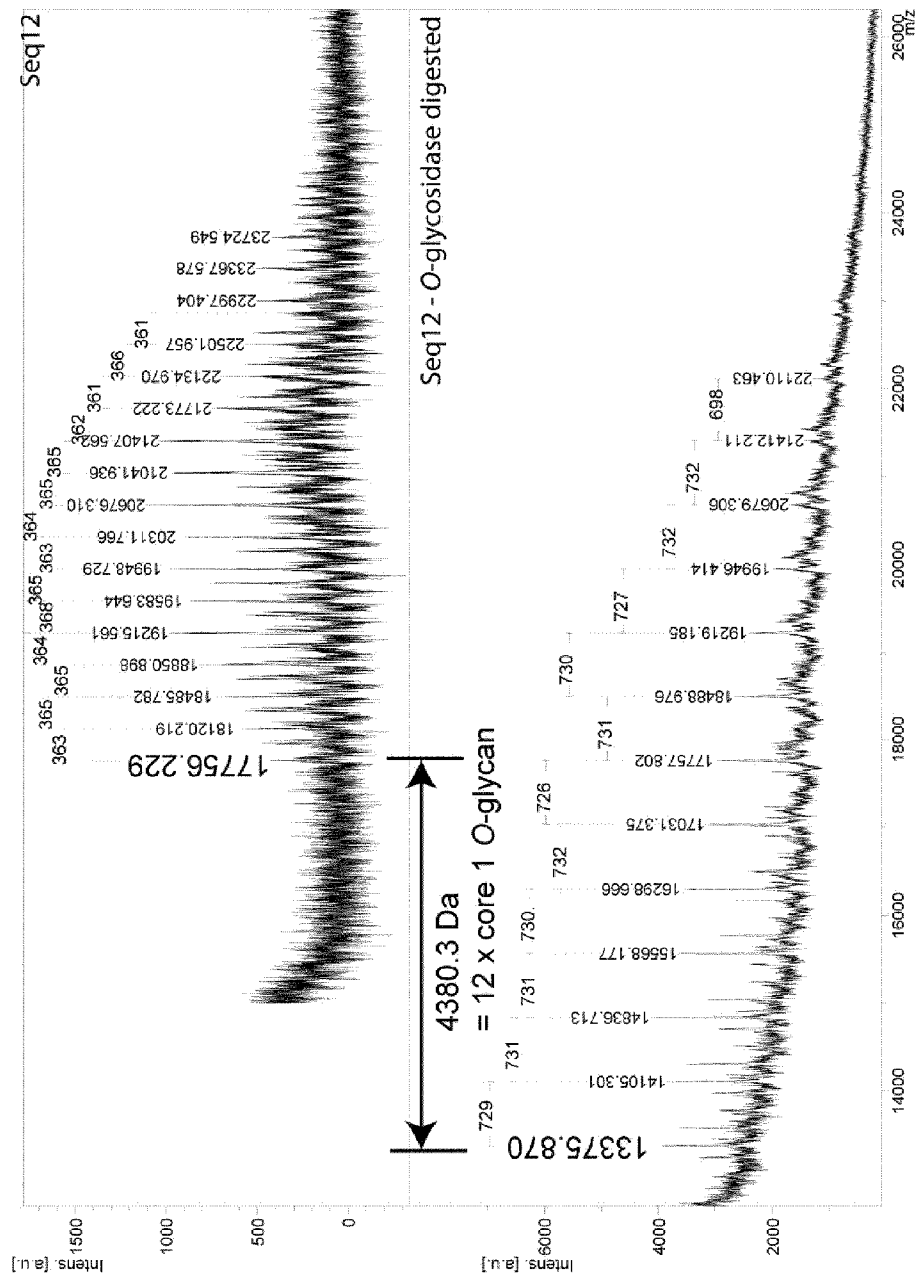


Fig. 9

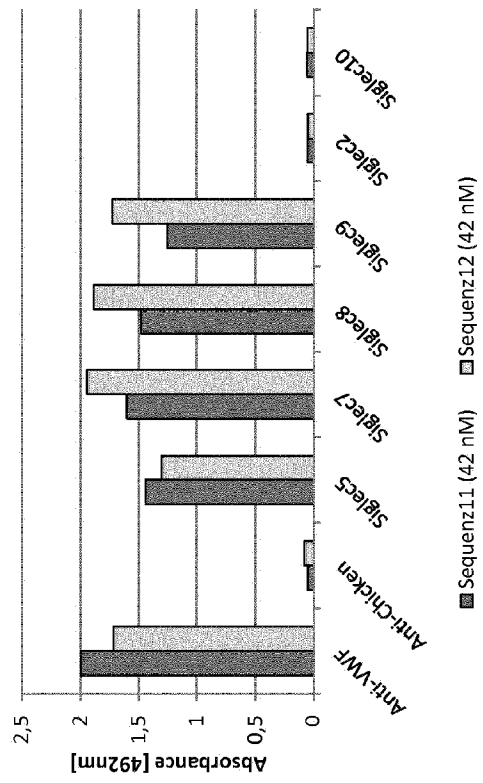
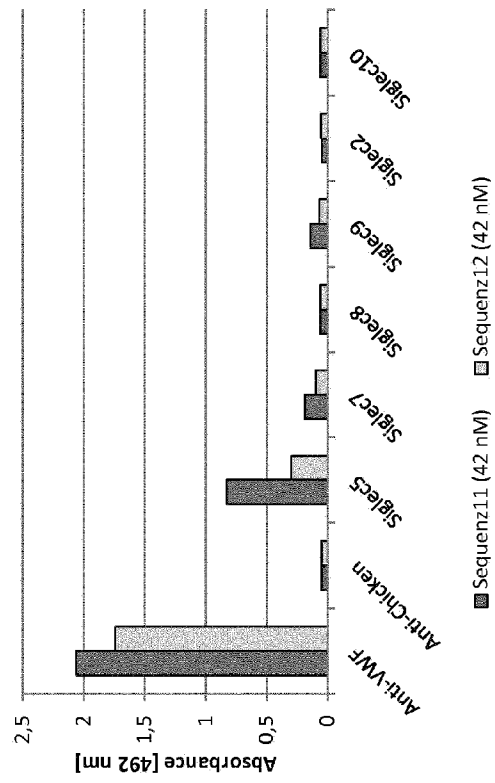


Fig. 10

**Fig. 11**

Seq11

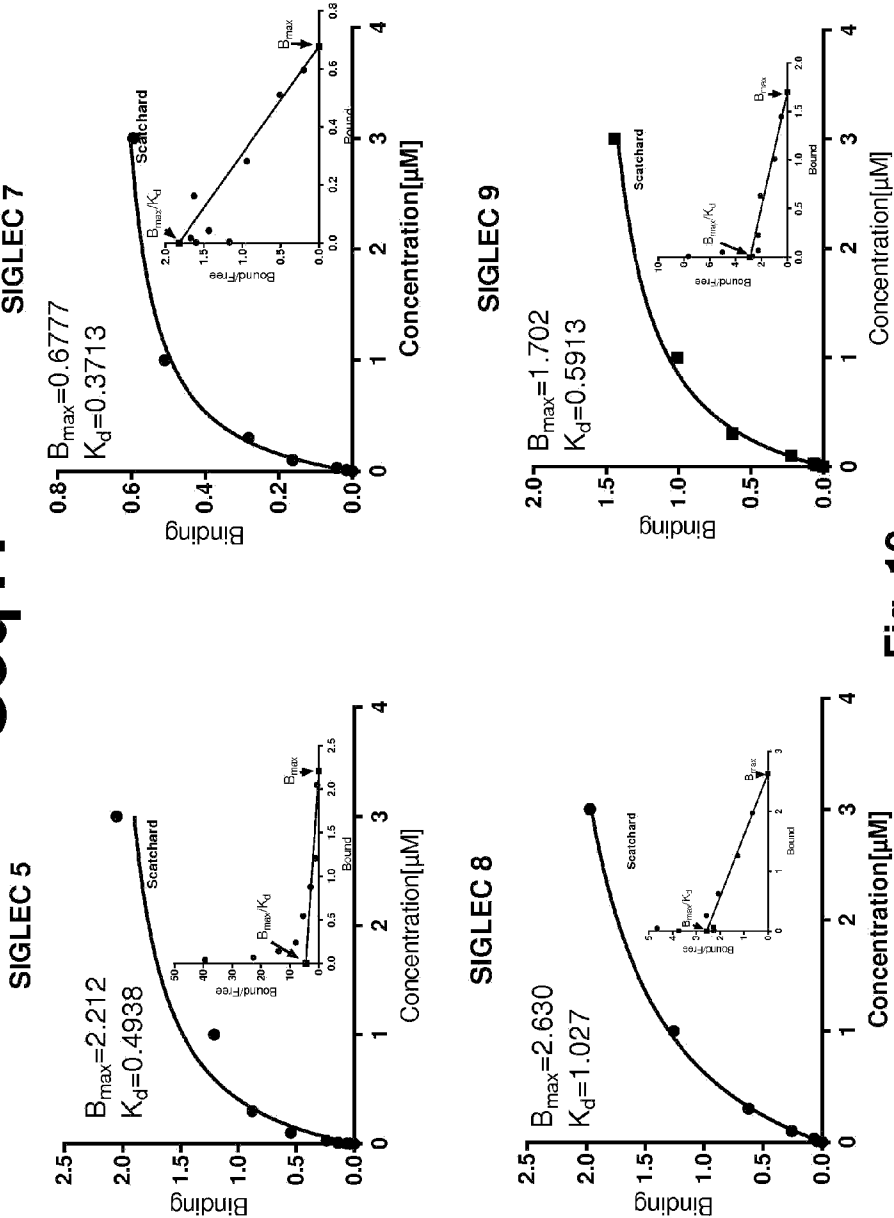
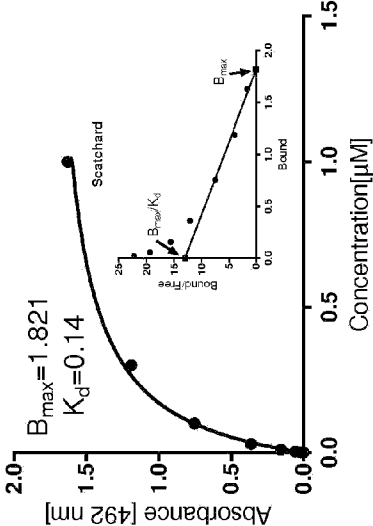


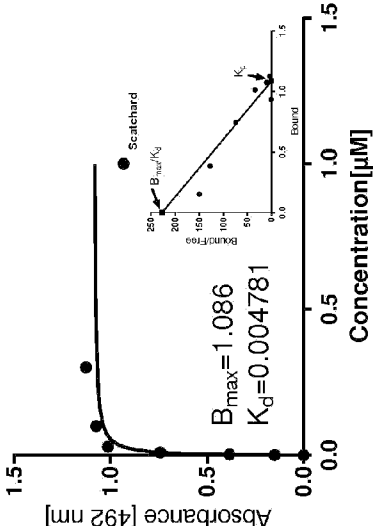
Fig. 12

Seq12

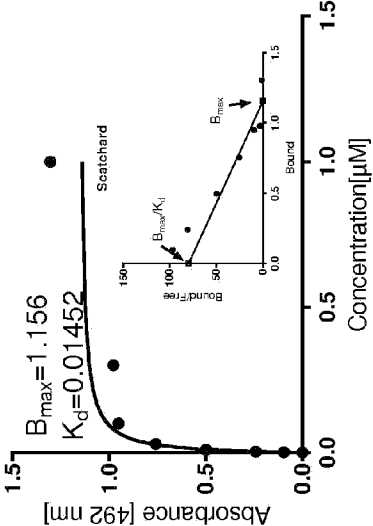
SIGLEC 5



SIGLEC 7



SIGLEC 8



SIGLEC 9

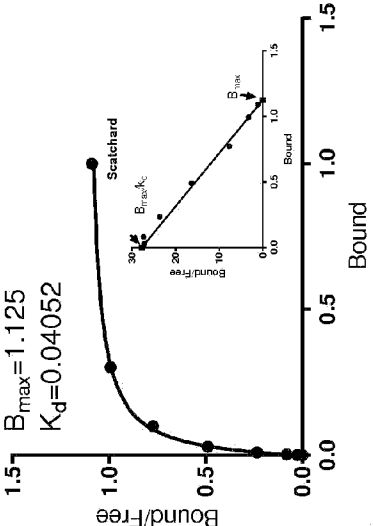


Fig. 13

	SIGLEC 5	SIGLEC 7	SIGLEC 8	SIGLEC 9
Seq11	0.494	0.371	1.027	0.591
Seq12	0.140	0.005	0.015	0.041

Estimated KD values based on Scatchard plots expressed in μM

Fig. 14

FVIII binding affinities mean $\pm$ -SD of five measurements

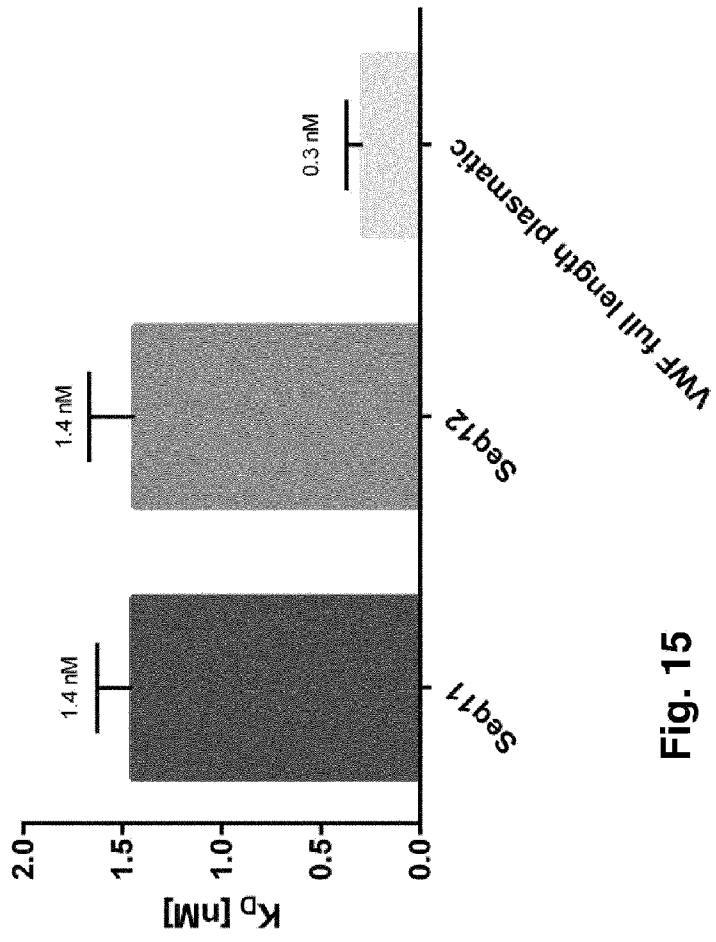


Fig. 15

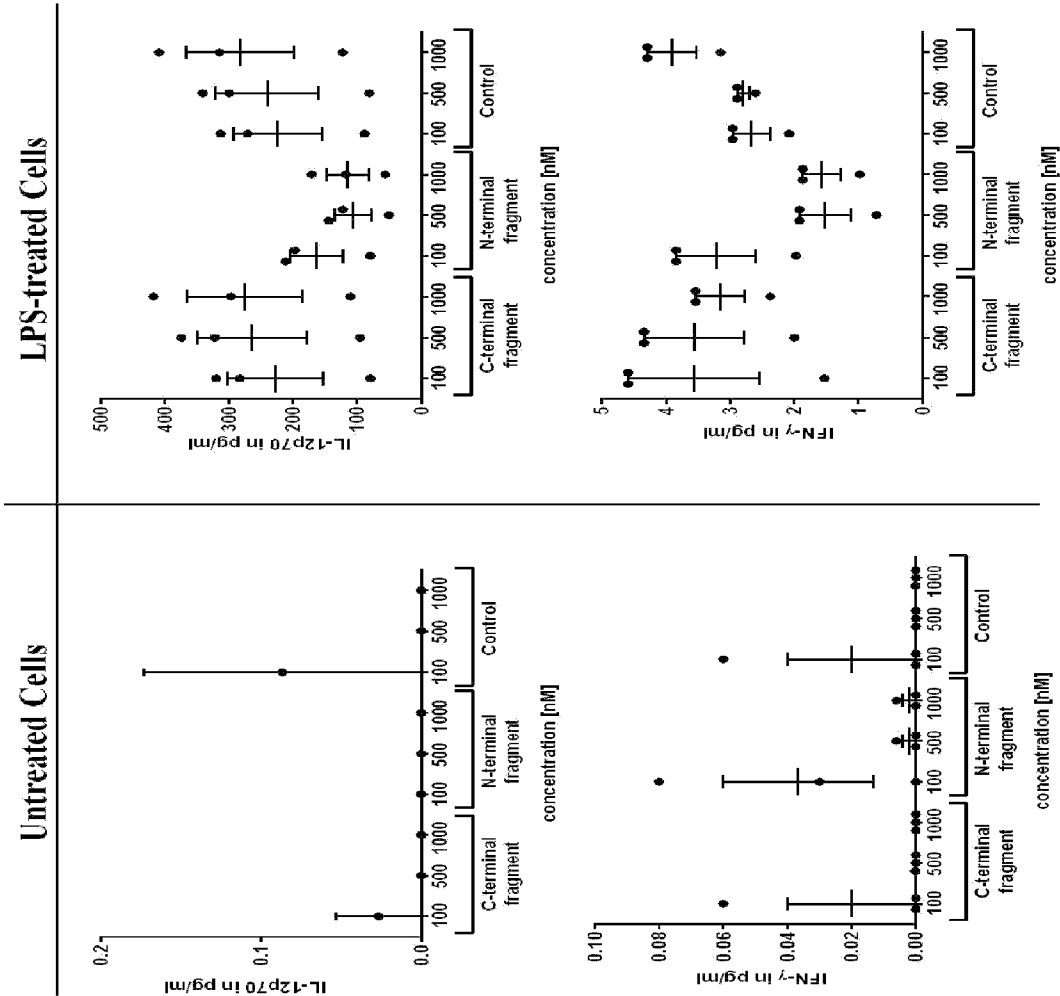


Fig. 16

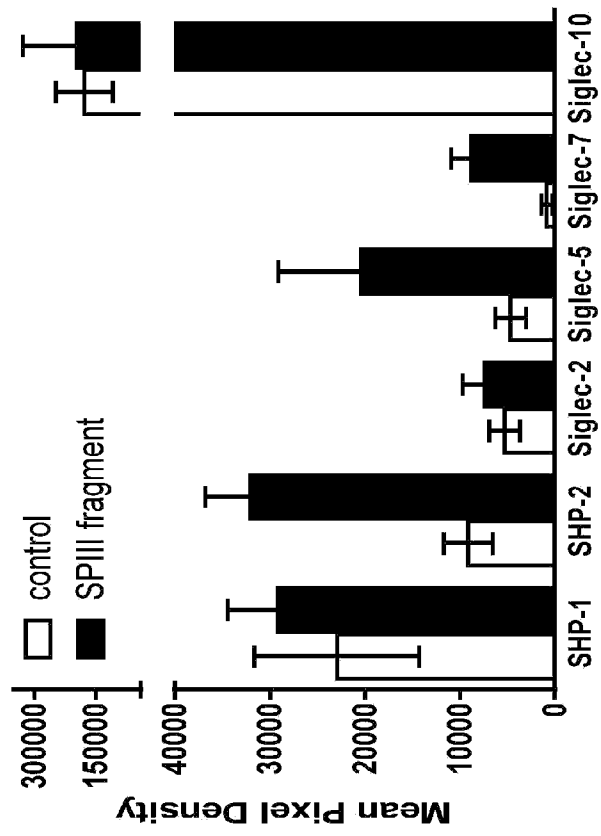


Fig. 17

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.

