(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 January 2016 (21.01.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/009232 A1

(51) International Patent Classification:
C07K 16/46 (2006.01) *A61K 39/395* (2006.01)

(21) International Application Number:
PCT/GB2015/052098

(22) International Filing Date:
20 July 2015 (20.07.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1412821.9 18 July 2014 (18.07.2014) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

WO 2016/009232 A1

(54) Title: POLYMERIC PROTEINS AND USES THEREOF

(57) Abstract: Provided are polymeric proteins that comprise two or more polypeptide monomer units, each monomer unit comprising two chimeric protein chains. Each chimeric polypeptide monomer unit comprises an Fc receptor binding portion comprising two immunoglobulin G heavy chain constant regions,wherein each immunoglobulin G heavy chain constant region comprises a cysteine residue which is linked via a disulfide bond to a cysteine residue of an immunoglobulin G heavy chain constant region of an adjacent polypeptide monomer unit. Each chimeric protein chain also comprises a modified immunoglobulin M tailpiece region, wherein the amino acid sequence of each chimeric polypeptide monomer comprises an alteration of the primary structure as compared to the native sequences from which the immunoglobulin G heavy chain constant region or immunoglobulin M tailpiece region are derived, and the alteration changes the number of glycosylation sites in a manner that promotes polymerisation. This promotion of polymerisation may lead to the generation of tetrameric, hexameric, and even dodecameric forms of the proteins. The proteins are suitable formedical uses,such as in the prevention or treatment of autoimmune diseases such as idiopathic thrombocytopenia. Also provided are methods of treatment using the polymeric proteins, and pharmaceutical compositions comprising the polymeric proteins.

POLYMERIC PROTEINS AND USES THEREOF

Field of the invention

The present invention relates to polymeric proteins. The invention also relates to the medical uses of such proteins, in particular in the prevention or treatment of autoimmune diseases such as idiopathic thrombocytopenia. The invention further relates to methods of treatment using polymeric proteins, and to pharmaceutical compositions comprising polymeric proteins.

Background

Autoimmune diseases (ADs) are common and affect 50 million American citizens alone. IVIG (purified immunoglobulin G) is one of the most common treatments of ADs, with Food and Drug Administration (FDA) approval for a diverse range of diseases like idiopathic thrombocytopenia (ITP), Kawasaki disease, Guillain-Barré, dermatomyositis, and chronic inflammatory demyelinating polyneuropathy.

As 70% of the global supply (worth $5 billion in 2012) of IVIG is now used to treat ADs, it is increasingly becoming unavailable to patients that need it most, in particular individuals with primary immune deficiency where IVIG is used as replacement therapy.

The worldwide consumption of IVIG has increased over 300 fold since 1980 and currently 100 ton are consumed per annum. Supplies of IVIG within the NHS and globally are critically limited, meaning that patients with an urgent need for the drug are routinely deprived of it (see letters of support from ITP support group, IVIG makers & NHS users). There are also significant clinical limitations resulting from its dependence on human donors for manufacture, and from the fact that <5% of injected IVIG (correctly glycosylated and/or oligomeric-Fc) is therapeutically active leading to a requirement for high doses (2g/kg) when used in ITP. Consequently, IVIG is expensive and adverse events due to excessive protein loading not uncommon.

Summary of the invention

In a first aspect the invention provides a polymeric protein comprising two or more polypeptide monomer units, each monomer unit comprising two chimeric protein chains;

wherein each chimeric polypeptide monomer unit comprises an Fc receptor binding portion comprising two immunoglobulin G heavy chain constant regions;
wherein each immunoglobulin G heavy chain constant region comprises a cysteine residue which is linked via a disulfide bond to a cysteine residue of an immunoglobulin G heavy chain constant region of an adjacent polypeptide monomer unit; and
and each chimeric protein chain also comprises a modified immunoglobulin M tailpiece region;
wherein the amino acid sequence of each chimeric polypeptide monomer comprises an alteration of the primary structure as compared to the native sequences from which the immunoglobulin G heavy chain constant region or immunoglobulin M tailpiece region are derived, and the alteration changes the number of glycosylation sites in a manner that promotes polymerisation.

In a second aspect the invention provides a polymeric protein according to the first aspect of the invention for use as a medicament.

In a third aspect the invention provides a polymeric protein according to the first aspect of the invention for use as a medicament in the prevention or treatment of an autoimmune or inflammatory disease.

In a fourth aspect the invention provides a pharmaceutical composition comprising a polymeric polypeptide according to the first aspect of the invention and a pharmaceutically acceptable carrier.

In a fifth aspect the invention provides a method of preventing or treating an autoimmune or inflammatory disease, the method comprising providing a subject in need of such treatment with a therapeutically effective amount of a polymeric protein of the invention.

In a sixth aspect the invention provides a method of intravenous immunoglobulin (IVIG) therapy, the method comprising providing a subject in need of such treatment with a therapeutically effective amount of a polymeric protein of the invention.

Detailed description of the invention

The present invention is based upon the inventors' surprising finding that alterations to the number of glycosylation sites within the primary sequence of chimeric proteins produced

by combining sequences of IgG and IgM are able to significantly impact upon the propensity of these proteins to polymerise. In particular, altering the number of glycosylation sites within such proteins is able to promote polymerisation. This promotion of polymerisation may be manifest in the generation of tetrameric, hexameric, and even dodecameric forms of the proteins.

The sequence of the tailpiece of IgM has previously been modified in a manner that prevented attachment of glycan at Asn563 of IgM. This modification resulted in enhanced polymer formation, evident in an increase in the number of hexamers produced by such modified proteins as compared to the number of pentamers. However, the inventors were surprised to find that the extent of polymerisation in polypeptides of the invention far exceeded that which might be expected based upon previously available information. Indeed in illustrative examples of the polypeptides of the invention >95% of the protein is secreted as a discrete dodecameric species, with no hexamers, pentamers or tetramers observed. The inventors believe that the production of immunoglobulin-based dodecamers, and certainly such production at the high levels observed in the present case, is a property that is, as yet, unique to the polymeric proteins of the invention.

The increase in polymerisation observed, in addition to being surprising, provides a number of advantages not found in the proteins of the prior art.

As long as that they meet the requirement of including an alteration of the primary structure that changes the number of glycosylation sites in a manner that promotes polymerisation (specific examples of which are considered in more detail below), the polypeptides of the invention may incorporate further variations from the native sequences of the IgG and/or IgM from which they are derived. Merely by way of example, a suitable polymeric protein of the invention may utilise IgG and/or IgG derived sequences that share at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the relevant native IgG or IgM sequence from which they are derived.

In a suitable embodiment, the immunoglobulin G heavy chain constant regions employed in the polymeric proteins of the invention are derived from an immunoglobulin selected from the group consisting of: IgG1; IgG2; IgG3; and IgG4. In particular, the immunoglobulin G heavy chain constant regions may be derived from IgG1.

The chimeric protein monomers may comprise residues 221 to 447 of human IgG1 combined with residues 561 to 575 of the tailpiece of human IgM.

These immunoglobulin sequences that make up the chimeric proteins naturally include two sites that undergo N-linked glycosylation, namely the asparagine corresponding to residue 297 of the Fc of IgG1 (Asn297) and the asparagine corresponding to residue 563 of IgM (Asn563).

It will be appreciated that in chimeric proteins, where not all of the IgG or IgM sequences are present, numbering of residues based upon the full-length native molecules is no longer informative. Accordingly, we will also refer in this disclosure to a reference chimeric protein sequence, which is set out as SEQ ID NO:1. This sequence represents a single chimeric polypeptide chain from within a monomer unit. When referring to this sequence Asn297 of IgG1 is renumbered as Asn77 of the fusion protein (the 77^{th} residue of SEQ ID NO:1) and Asn563 of IgM is renumbered as Asn236 (the 236^{th} residue of the SEQ ID NO:1).

For the avoidance of doubt, a polymeric protein consisting of chimeric protein chains having the sequence set out in SEQ ID NO:1 will not constitute a polymeric protein of the invention, since it will not incorporate the requisite alteration in primary structure.

The IgG and IgM-derived portions of the chimeric proteins may be joined via a linker sequence. The inventors have found that a 6 amino acid linker sequence Leu-Val-Leu-Gly-Pro-Pro inserted between residue K447 of IgG and residue L450 of the tailpiece facilitates polymerisation, since it allows cysteine residues in the tailpiece to associate more closely with cysteine residues in adjacent monomers, thereby increasing the formation of covalent bonds. Accordingly, the polypeptides of the invention may incorporate a six amino acid linker between the IgG and IgM derived sequences. Suitably the six amino acid linker may comprise Leu- Val-Leu-Gly-Pro-Pro. This linker is present in the reference chimeric protein, where it facilitates the involvement of Cys248 of SEQ ID NO:1 (corresponding to Cys575 in IgM) in covalent bonds.

In a suitable embodiment the alteration of the primary structure that changes the number of glycosylation sites comprises disruption of the glycosylation site corresponding to Asn297 in the Fc of IgG1, or disruption of the glycosylation site corresponding to Asn563 of IgM. Such disruption may decrease the total number of Disruption of the glycosylation

sites may be achieved by substitution of any of the residues that are required in the asparagine-X-serine/threonine motif at which N-glycosylation occurs. It will be appreciated that any of these residues may be replaced with any residue (either naturally occurring or non-naturally occurring) that disrupts the motif. Suitably the asparagine residue (Asn) may be substituted with an alanine residue (Ala).

Additionally or alternatively, and as discussed further below, the alteration of the primary structure that changes that number of glycosylation sites in a manner that promotes polymerisation may comprise the addition of a further glycosylation site not found in the native sequences from which the fusion protein has been derived. Examples of such polypeptides of the invention include those, such as SEQ ID NOs: 5, 6, and 7 that incorporate the introduction of an Asn residue at the N-terminal (a change to residue 1 of SEQ ID NO:1 also referred to herein as D001N). This causes the formation of a new glycosylation motif that is not found in SEQ ID NO:1.

Returning to proteins of the invention in which glycosylation sites are disrupted, for the purposes of the present disclosure references to polymeric proteins that include a disruption of the glycosylation site corresponding to Asn297 in the Fc of IgG1, or disruption of the glycosylation site corresponding to Asn563 of IgM, should be taken as limited to those polymeric proteins in which only one of these glycosylation sites has been disrupted. Such a definition does not encompass polymeric proteins in which both the glycosylation site corresponding to Asn297 in the Fc of IgG1 and the glycosylation site corresponding to Asn563 of IgM have been disrupted.

In a suitable embodiment of the polymeric protein described in the preceding paragraph, where the polymeric protein is based upon the sequence set out in SEQ ID NO:1, the alteration may comprise disruption of the glycosylation site corresponding to Asn77 of SEQ ID NO:1 or of the glycosylation site corresponding to Asn236 of SEQ ID NO:1. Once again, such a definition would not encompass polymeric proteins based upon SEQ ID NO:1 in which both the glycosylation sites at Asn77 and Asn236 of SEQ ID NO:1 are disrupted.

This stipulation that the sequence comprise an alteration at one or other of the glycosylation sites found in the fusion protein is based upon the inventor's surprising finding that, while disruption of either of these glycosylation sites is able to promote polymerisation, this effect is not additive. Indeed, as described in the Examples of the

present application, polypeptides in which both the glycosylation site at Asn297 of IgG/Asn77 of SEQ ID NO:1 and the glycosylation site at Asn563 of IgM/Asn 236 of SEQ ID NO:1 have been disrupted (such as those of SEQ ID NO:4 or SEQ ID NO:8) do not polymerise at all, and so do not constitute polymeric proteins of the invention.

In a suitable embodiment the alteration of the primary structure comprises an alteration at a residue corresponding to Asn563 of IgM. In the case of polymeric proteins based upon SEQ ID NO:1, this alteration of the primary structure comprises an alteration at a residue corresponding to Asn236 of SEQ ID NO:1. As illustrated further in the Examples set out below, the inventors have found that polymeric proteins comprising this alteration have particularly surprising and beneficial properties. In particular, these proteins exhibit much higher levels of polymerisation than have previously been described in the scientific literature. For example, the polymeric proteins of SEQ ID NO:3 and SEQ ID NO:7, both of which incorporate a substitution of alanine for Asn236 of SEQ ID NO:1, both exhibit a tendency to form dodecamers. Furthermore the inventors have found that in conditions allowing polymerisation, dodecamers constitute >99% of the total polymers present, without significant quantities of other polymeric intermediaries (e.g. hexamers, pentamers as seen with IgM). This remarkable level of homogeneity is particularly advantageous for therapeutic applications, where a homogeneous product is important to ensure consistency of dosing, and hence of therapeutic activity.

While SEQ ID NO:3 differs from SEQ ID NO:1 only in respect of the substitution of Asn236 with an alanine residue, SEQ ID NO:7 is an example of a polymeric protein of the invention in which a further glycosylation site has been added, specifically by the introduction of an asparagine residue at the N-terminal (corresponding to residue 1 of SEQ ID NO:1) which yields an additional glycosylation site.

In a suitable embodiment, a polymeric protein according to this aspect of the invention may comprise a sequence selected from the group consisting of: SEQ ID NO:3; and SEQ ID NO:7.

Suitably a polymeric protein according to this embodiment of the invention comprises SEQ ID NO:3, which is to say that the polymeric protein incorporates the sequence of SEQ ID NO:3, and optionally further amino acid residues.

Suitably a polymeric protein according to this embodiment of the invention comprises SEQ ID NO:7. As with the preceding paragraph, this definition should be taken as encompassing a polymeric protein that incorporates the sequence of SEQ ID NO:7, and optionally further amino acid residues.

In a suitable embodiment, a polymeric protein according to this aspect of the invention may consist of a sequence selected from the group consisting of: SEQ ID NO:3; and SEQ ID NO:7. Thus, in one embodiment a polymeric protein of the invention consists of monomer units of SEQ ID NO:3. In another embodiment a polymeric protein of the invention consists of monomer units of SEQ ID NO:7.

Polymeric proteins of the invention may alternatively comprise an alteration at a residue corresponding to Asn297 in the Fc of IgG1. In the case of a polymeric protein based upon SEQ ID NO:1, this comprises an alteration at a residue corresponding to Asn77 of SEQ ID NO:1.

Examples of such polymeric proteins of the invention are provided in SEQ ID NO:2 and SEQ ID NO:6. SEQ ID NO:2 differs from SEQ ID NO:1 only in that Asn77 has been substituted by an alanine residue. SEQ ID NO:6 is a further example of a polymeric protein of the invention in which a further glycosylation site has been added. Once again (as with SEQ ID NO:7 discussed above), this has been achieved by the introduction of an asparagine residue at the N-terminal (corresponding to residue 1 of SEQ ID NO:1) which yields an additional glycosylation site not found in the corresponding portion of native IgG1.

Both proteins made up of SEQ ID NO:2 and those made up of SEQ ID NO: 6 exhibit high levels of polymerisation – with both proteins being found predominantly in the hexameric form (see the Examples).

In a suitable embodiment, a polymeric protein according to this aspect of the invention may comprise a sequence selected from the group consisting of: SEQ ID NO:2; and SEQ ID NO:6.

Suitably a polymeric protein according to this embodiment of the invention comprises SEQ ID NO:2. Alternatively a polymeric protein according to this embodiment of the invention may comprise SEQ ID NO:6.

In a suitable embodiment, a polymeric protein according to this aspect of the invention may consist of a sequence selected from the group consisting of: SEQ ID NO:2; and SEQ ID NO:6. In one suitable embodiment a polymeric protein of the invention consists of monomer units of SEQ ID NO:2. In another embodiment a polymeric protein of the invention consists of monomer units of SEQ ID NO:6.

As has been referred to above, the alteration of the primary structure that changes that number of glycosylation sites in a manner that promotes polymerisation may comprise the addition of a further glycosylation site not found in the native sequences from which the fusion protein has been derived. While in SEQ ID NO:6 and SEQ ID NO:7 such a change is found in combination with a disruption of one or other of the naturally occurring glycosylation sites in the IgG or IgM sequences from which the fusion protein is derived, in a suitable embodiment the addition of a further glycosylation site in this manner may be the only alteration, and may still serve to promote polymerisation.

Merely by way of example, the fusion protein of SEQ ID NO:5 retains both Asn77 and Asn236 of SEQ ID NO:1, while adding a further glycosylation site at the N-terminus of the fusion protein through the substitution of the naturally occurring aspartic acid residue with an asparagine residue. This alteration of the primary sequence is again associated with the promotion of polymerisation, and proteins made up of SEQ ID NO:5 are found in hexameric form.

In each of SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7, substitution of aspartic acid at position 1 of SEQ ID NO:1 with an asparagine residue causes the addition of a glycosylation site in the hinge region of the immunoglobulin G heavy chain constant region. Glycosylation of the hinge region of antibodies is not found in nature, and it would be expected that this would interfere with disulphide bond formation. Since disulphide bond formation is necessary for the production of homodimers associated with formation of the Fc receptor binding portion, it is surprising to find that these polymeric proteins of the invention in which glycosylation occurs within the hinge region are biologically active.

The various specific polypeptide monomers referred to above, and their relationship to the reference sequence SEQ ID NO:1, are detailed further in Table 3, along with details of two comparator proteins (SEQ ID NO: 4 and SEQ ID NO:8) that are not of the invention, but

were produced by the inventors to further investigate and demonstrate the surprising and inventive properties of the peptides of the invention.

The polymeric proteins of the invention also encompass fragments or variants of the specific polypeptide monomer units set out in SEQ ID NO:2, SEQ ID NO:3; SEQ ID NO:5; SEQ ID NO:6; and SEQ ID NO:8 that share the biological activity of these "parent" polypeptides. In particular the fragments or variants should share at least the increased tendency towards polymerisation of the parent polypeptide from which they are derived. Suitably these fragments or variants may also share the activities discussed further below that make the parent polymeric proteins suitable for use in therapeutic applications.

A fragment of a polypeptide monomer unit set out in SEQ ID NO:2, SEQ ID NO:3; SEQ ID NO:5; SEQ ID NO:6; or SEQ ID NO:8 should include the alteration of the primary structure found in the parent polypeptide.

A variant of a polypeptide monomer unit set out in SEQ ID NO:2, SEQ ID NO:3; SEQ ID NO:5; SEQ ID NO:6; or SEQ ID NO:8 should also include the alteration of the primary structure found in the parent polypeptide. Other than that, a variant may share 70% or more identity with the parent polypeptide; 80% or more identity with the parent polypeptide; 90% or more identity with the parent polypeptide; 95% or more identity with the parent polypeptide; 96% or more identity with the parent polypeptide; 97% or more identity with the parent polypeptide; 98% or more identity with the parent polypeptide; or even 99% or more identity with the parent polypeptide. A variant may differ from the parent polypeptide by 1% or more, 2% or more, 3% or more, 4% or more, 5% or more, 10% or more, 20% or more, or even 30% or more with reference to sequence identity.

A suitable example of the variants considered above is a polymeric protein of the invention in which the position of one or more of the glycosylation sites set out in the polypeptide sequences of SEQ ID NOs: 2, 3, 5, 6, or 7 is "moved" as compared to these illustrative sequences.

Merely by way of example, a variant in accordance with this embodiment may have the Asn normally found at position 77 of SEQ ID NO:1 moved to a position corresponding to any of residues 72, 73, 74, 75, 76, 78, 79, 80, 81, or 82 (with the other associated residues of the glycosylation site undergoing a corresponding move). Alternatively or additionally, such a variant may have the Asn at position 236 of SEQ ID NO:1 moved to a

position corresponding to any of residues 231, 232, 233, 234, 235, 237, 238, 239, 240, or 241 (with the other associated residues of the glycosylation site undergoing a corresponding move). A further alternative or additional modification in a variant in accordance with this embodiment of the invention may involve the inclusion of a glycosylation site in which the Asn residue is positioned at a residues corresponding to any of 2, 3, 4, 5, or 6 of SEQ ID NO:1.

The inventors believe that the changes discussed above may affect the valency and/or induce a more uniform product (e.g. all polymers of the same size, as opposed to a range of polymer sizes). By moving the position of glycosylation sites up or down the molecule, glycan processing is affected because the peptide backbone constrains the molecular space available for glycan to be accommodated. This may also affect the nature of the glycans attached, such that these modifications may favour the addition of oligomannose type glycans over complex-types, and thus also affect interactions with effectors, as considered elsewhere in this disclosure.

The proteins of the invention also have biological activities that go beyond their ability to exhibit high levels of polymerisation. It is these properties, and their therapeutic applications, that give rise to the second, third, fourth, fifth, and sixth aspects of the invention.

Many of the properties of the polymeric proteins of the invention indicate that that they will be able to influence biological activities in therapeutically effective ways. This is reflected in the second aspect of the invention, which provides a polymeric protein in accordance with the first aspect of the invention for use as a medicament.

Specific medical applications of the polymeric proteins of the invention are considered in more detail in the following paragraphs.

The third and fifth aspects of the invention respectively relate to medical use of the polymeric proteins of the invention in the prevention or treatment of an autoimmune or inflammatory disease, and methods of preventing or treating an autoimmune or inflammatory disease, in which a subject in need of such treatment is provided with a therapeutically effective amount of a polymeric protein.

Suitable autoimmune or inflammatory diseases for treatment include those that are treatable with intravenous immunoglobulin (IVIG). These may be diseases which are currently routinely treated with IVIG or in which IVIG has been found to be clinically useful, such as autoimmune cytopenias, Guillain-Barré syndrome, myasthenia gravis, anti-Factor VIII autoimmune disease, dermatomyositis, vasculitis, and uveitis (See, van der Meche FG *et al*, Lancet i, 406 (1984); Sultan Y *et al*, Lancet ii, 765 (1984); Dalakas MC *et al*, N. Engl. J. Med. 329, 1993 (1993); Jayne DR *et al*, Lancet 337, 1137 (1991); LeHoang P *et al*, Ocul. Immunol. Inflamm. 8, 49 (2000). IVIG is typically used to treat idiopathic thrombocytopenic purpura (ITP), Kawasaki disease, Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy (Orange *et al*, 2006, J Allergy Clin Immunol 117: S525-53). IVIG is also increasingly used to treat a diverse array of other autoimmune diseases which are non-responsive to mainstay therapies, including arthritis, diabetes, myositis, Crohn's colitis and systemic lupus erythematosus.

Autoimmune or inflammatory diseases suitable for treatment include autoimmune cytopenia, idiopathic thrombocytopenic purpura, rheumatoid arthritis, systemic lupus erythematosus, asthma, Kawasaki disease, Guillain–Barré syndrome, Stevens–Johnson syndrome, Crohn's colitis, diabetes, chronic inflammatory demyelinating polyneuropathy myasthenia gravis, anti-Factor VIII autoimmune disease, dermatomyositis, vasculitis, uveitis or Alzheimer's disease.

Conditions to be treated may include an inflammatory disease with an imbalance in cytokine networks, an autoimmune disorder mediated by pathogenic autoantibodies or autoaggressive T cells, or an acute or chronic phase of a chronic relapsing autoimmune, inflammatory, or infectious disease or process. In addition, other medical conditions having an inflammatory component are included, such as Amyotrophic Lateral Sclerosis, Huntington's Disease, Alzheimer's Disease, Parkinson's Disease, Myocardial Infarction, Stroke, Hepatitis B, Hepatitis C, Human Immunodeficiency Virus associated inflammation, adrenoleukodystrophy, and epileptic disorders especially those believed to be associated with postviral encephalitis including Rasmussen Syndrome, West Syndrome, and Lennox-Gastaut Syndrome.

Conditions to be treated may be hematoimmunological diseases, e.g., Idiopathic Thrombocytopenic Purpura, alloimmune/autoimmune thrombocytopenia, Acquired immune thrombocytopenia, Autoimmune neutropenia, Autoimmune hemolytic anemia, Parvovirus B19-associated red cell aplasia, Acquired antifactor VIII autoimmunity,

acquired von Willebrand disease, Multiple Myeloma and Monoclonal Gammopathy of Unknown Significance, Aplastic anemia, pure red cell aplasia, Diamond-Blackfan anemia, hemolytic disease of the newborn, Immune-mediated neutropenia, refractoriness to platelet transfusion, neonatal post-transfusion purpura, hemolytic uremic syndrome, systemic Vasculitis, Thrombotic thrombocytopenic purpura, or Evan's syndrome.

Alternatively, a neuroimmunological disease may be treated, e.g., Guillain-Barré syndrome, Chronic Inflammatory Demyelinating Polyradiculoneuropathy, Paraproteinemic IgM demyelinating Polyneuropathy, Lambert-Eaton myasthenic syndrome, Myasthenia gravis, Multifocal Motor Neuropathy, Lower Motor Neuron Syndrome associated with anti-GM1 antibodies, Demyelination, Multiple Sclerosis and optic neuritis, Stiff Man Syndrome, Paraneoplastic cerebellar degeneration with anti-Yo antibodies, paraneoplastic encephalomyelitis, sensory neuropathy with anti-Hu antibodies, epilepsy, Encephalitis, Myelitis, Myelopathy especially associated with Human T-cell lymphotropic virus-1, Autoimmune Diabetic Neuropathy, or Acute Idiopathic Dysautonomic Neuropathy or Alzheimer's disease.

A rheumatic disease may be treated, e.g., Kawasaki's disease, Rheumatoid arthritis, Felty's syndrome, ANCA-positive Vasculitis, Spontaneous Polymyositis, Dermatomyositis, Antiphospholipid syndromes, Recurrent spontaneous abortions, Systemic Lupus Erythematosus, Juvenile idiopathic arthritis, Raynaud's, CREST syndrome or Uveitis.

A dermatoimmunological disease may be treated, e.g., Epidermal Necrolysis, Gangrene, Granuloma, Autoimmune skin blistering diseases including Pemphigus vulgaris, Bullous Pemphigoid, and Pemphigus foliaceus, Vitiligo, Streptococcal toxic shock syndrome, Scleroderma, systemic sclerosis including diffuse and limited cutaneous systemic sclerosis, Atopic dermatitis or steroid dependent Atopic dermatitis.

A musculoskeletal immunological disease may be treated, e.g., Inclusion Body Myositis, Necrotizing fasciitis, Inflammatory Myopathies, Myositis, Anti-Decorin (BJ antigen) Myopathy, Paraneoplastic Necrotic Myopathy, X-linked Vacuolated Myopathy, Penacillamine-induced Polymyositis, Atherosclerosis, Coronary Artery Disease, or Cardiomyopathy.

A gastrointestinal immunological disease may be treated, e.g., pernicious anemia, autoimmune chronic active hepatitis, primary biliary cirrhosis, Celiac disease, dermatitis

herpetiformis, cryptogenic cirrhosis, Reactive arthritis, Crohn's disease, Whipple's disease, ulcerative colitis or sclerosing cholangitis.

The disease can be, for example, post-infectious disease inflammation, Asthma, Type 1 Diabetes mellitus with anti-beta cell antibodies, Sjogren's syndrome, Mixed Connective Tissue Disease, Addison's disease, Vogt-Koyanagi-Harada Syndrome, Membranoproliferative glomerulonephritis, Goodpasture's syndrome, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, micropolyarterits, Churg-Strauss syndrome, Polyarteritis nodosa or Multisystem organ failure.

An exemplary disease for treatment in idiopathic thrombocytopenic purpura (ITP).

In a sixth aspect the invention provides a method of intravenous immunoglobulin (IVIG) therapy, the method comprising providing a subject in need of such treatment with a therapeutically effective amount of a polymeric protein of the invention. It will be appreciated that IVIG treatment is of benefit to many patients subject to autoimmune or inflammatory diseases.

Polymeric proteins of the invention for use in IVIG may be selected on the basis of increased avidity of binding to inhibitory glycan- and Fc-receptors involved in controlling pro-inflammatory responses at the expense of binding pro-inflammatory glycan receptors that lead to enhanced clearance and potential adverse events. Polymeric proteins of the invention including an additional glycosylation site at the position corresponding to residue 1 of SEQ ID NO:1 exhibit decreased complement activation and this may be desirable although removal of complement binding sites e.g. Lys322 to Ala could also be contemplated if complement activation is not desirable in the final medicament.

The fourth aspect the invention provides pharmaceutical compositions comprising a polymeric polypeptide of the invention and a pharmaceutically acceptable carrier.

Suitable additional constituents of that may be included in a pharmaceutical composition of the invention include excipients, diluents, buffering agents and preservatives. Examples of such additional constituents will be apparent to those of skill in the art on consideration of the use to which the composition is to be put, and the desired route of administration.

The following paragraphs provide details of certain considerations that may be used in determining which polymeric proteins of the invention are suitable for use in the various therapeutic applications set out above.

The increased extent of polymer formation on the part of polymeric proteins of the invention makes them very suitable for use as Fc-fusion medicines. Polymeric proteins of the invention suitable for use in accordance with these embodiments will further comprise at least one therapeutic moiety associated with the monomer units. Suitably, the polymeric proteins of the invention may comprise at least one therapeutic moiety associated with each of the chimeric protein chains. The therapeutic moiety may be fused to, or otherwise associated with, the chimeric protein chains. Since the polymeric proteins of the invention are able to produce dodecamers comprising 24 chimeric protein chains, if each of these is associated with a therapeutic moiety then very large numbers of such moieties may be provided by the molecules of the invention. Furthermore, this can be achieved without loss of binding to critical receptors e.g. DC-SIGN and complement.

Therapeutic moieties that may be attached to the polymeric proteins of the invention include drugs, toxins and other cleavable payloads.

The polymeric proteins of the invention also lend themselves to use in improved vaccines. The large numbers of antigen binding sites present in the polymeric proteins of the invention means that they are able to increase the number of antigens delivered to dendritic cells (DCs) – thus increasing the depot effect of adjuvants. The binding of polymeric proteins of the invention (such as those comprising SEQ ID NO:3, also referred to as N236A) to monocytes and DC-SIGN illustrates their properties that are highly attractive with respect to vaccine use.

The capacity of the polymeric proteins of the invention to bind to the FcRn may be altered by modification of the residue corresponding to 310 of SEQ ID NO:1. In cases where it is desired that the polymeric proteins should exhibit FcRn binding (for example in cases where long half-life of the proteins in the blood of a subject is required after injection) a histidine residue may be included at the position corresponding to residue 310 of SEQ ID NO:1. Alternatively, in cases where FcRn binding is not considered desirable (for example in applications where the polymeric protein is to be injected into the eye and it is wished that it will maintain this location) the residue corresponding to position 301 of SEQ ID NO:1 may be one such as leucine.

Furthermore, the invnetors found that addition of a glycosylation site at a position corresponding to residue 1 of SEQ ID NO:1 led to a significant decrease in the ability of proteins to activate complement as compared to SEQ ID NO:1 itself (which lacks this glycosylation site).

These findings demonstrate that polymeric proteins comprising SEQ ID NO:2, 6, or 7 may be utilised in contexts where complement activation may be disadvantageous, such as in IVIG for treatment of autoimmune disease.

Polymeric proteins of the invention in which the glycosylation site corresponding to Asp 77 of IgG has been disrupted exhibit a reduction in their interactions with mannose dependent receptors, as exemplified by DC-SIGN. In contrast, when the glycosylation site in the sequence derived from the IgM tailpiece (at residue 236 of SEQ ID NO:1) was disrupted this did not hinder DC-SIGN binding of proteins incorporating this change. Furthermore polymers in which Asp 77 was mutated to Ala, but where the N236 glycan was left intact, were unable to interact with DC-SIGN further enforcing the importance of the N77 glycan to DC-SIGN binding.

This important finding shows that it is the oligomannose type glycans found on N77 that contribute to binding to mannose dependent receptors such as DC-SIGN. It has previously been thought that oligomannose type glycans are associated with the glycosylation site present in the tailpiece of IgM, and so this inventors' finding that glycosylation within the IgG-derived sequence (for example at Asp77 of SEQ ID NO:1) appears to be key to interaction with mannose dependent receptors is highly surprising.

In addition the binding of polymeric proteins of the invention to mannose-dependent receptors (again exemplified by DC-SIGN) can be modulated by the insertion of additional glycans, as seen with polymeric proteins of the invention that incorporate the addition of a new glycosylation site (for example at D001N of SEQ ID NO:1).

For example binding of dodecameric polypeptides of SEQ ID NO:7 (also referred to as D001N/N236A) mutant to DC-SIGN was significantly reduced (>50%) by comparison with the binding of dodecamers of SEQ ID NO:3 (also referred to as N236A).

A similar reduction (approximately 50%) in binding to DC-SIGN was observed when comparing binding of hexameric proteins of the invention comprising SEQ ID NO:5 (also referred to as D001N) with binding of hexameric comparator proteins of SEQ ID NO:1.

When the inventors compared the ability of dodecameric proteins of the invention and hexameric proteins of SEQ ID NO:1 to bind to DC-SIGN, they found that the dodecamers did not exhibit increased binding. This suggests that increasing the valency from 6 to 12 does not lead to greater interactions with DC-SIGN.

It is known that mannose-binding receptors, such as the mannose receptor (MR), are associated with pro-inflammatory responses, and that oligomannose type glycans found on IgG are associated with enhanced pro-inflammatory effector functions and rapid *in vivo* clearance (for example by splenic macrophages) of the molecules to which they are attached.

The present finding suggests that polypeptides of the invention in which the glycosylation site corresponding to N77 of SEQ ID NO:1 is disrupted and/or an additional glycosylation site (such as at residue 1 of SEQ ID NO:1) is added may have decreased tendency to induce inflammation. They may also have improved half-lives and PK, particularly when used as a biomimetic of IVIG for the treatment of autoimmune disease. Furthermore, the finding that increased valency does not correspond to increased induction of inflammation suggests that embodiments of the proteins of the invention that are fused to other therapeutic molecules (for example for the delivery of a payload of such drugs), may achieve improved yield of a fused therapeutic agent without a concomitant increase in adverse events associated with mannose-dependent receptor-mediated inflammation.

These findings also lend themselves to applications in embodiments in which interactions with mannose binding receptors are desirable, such as when delivering antigens in vaccines. In such embodiments it may be wished to use a polymeric protein of the invention in which the glycosylation site corresponding to N77 of SEQ ID NO:1 has been retained, and also to modify the polymeric proteins to enhance oligomannose attachment to N77.

The glycans associated with these glycosylation sites in the polymeric proteins of the invention may be modified by selection of the cell type in which the proteins are expressed. Chinese hamster ovary (CHO) cell lines deficient for various glycosylation

enzymes are available, and expression of the proteins of the invention in these cell lines is able to yield proteins with a desired glycan composition.

Further details of CHO cell lines with glycosylation enzyme deficiencies are found in North *et al.* "Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals *N*-glycans of a novel size and complexity" (J Biol Chem 285(8):5759–5775). Merely by way of example, expression of polymeric proteins of the invention in lec1 or lec3.2.8.1 modified CHO cells will be expected to yield polypeptides richer in Mann5, Mann9 glycans. The presence of such glycans may improve interactions of the polymeric proteins with DC-SIGN or other lectin receptors.

It has also been suggested that terminal sialic acid is involved in DC-SIGN binding, and it is known that growing CHO cells as monolayers (rather than in suspension) is able to enhance sialylation. Thus, in the case that it is wished to enhance the interaction of polymeric proteins of the invention with DC-SIGN the proteins may be expressed by CHO cells grown in monolayer culture, and in the case that it is wished to decrease the interaction of polymeric proteins of the invention with DC-SIGN the proteins may be expressed by CHO cells grown in suspension culture.

Galactose may bind dectin-1, and so in the case that it is wished to produce polymeric proteins of the invention with enhanced binding to dectin-1, these proteins may be expressed in lec4 mutant CHO cell lines. This expression will be expected to lead to bi- and tri-antennary structures fully capped with galactose. Such proteins may promote the association of FcγRIIb with dectin-1, thereby blocking C5a-dependent inflammation *in vivo*. Such proteins of the invention may therefore be of utility in the prevenion and/or treatment of inflammatory condidtions including peritonitis and skin blistering in epidermolysis bullosa (for example by means of IVIG treatment, which is already known to positively affect these conditions).

Definitions

Various terms used in the present disclosure will now be further described with reference to the following definitions.

"Polymers" and "monomers"

The polymeric proteins are composed of multiple polypeptide units referred to as "monomers" or "monomer units" for the purposes of the present disclosure. Each of these monomer units comprises two disulphide bonded chains, each chain being a chimeric protein formed by combining sequence based upon an immunoglobulin G heavy chain and sequence based upon a modified tailpiece region from immunoglobulin M.

Thus, in the context of the present invention, a "pentamer" will comprise 5 "monomers" for a total of 10 disulphide bonded chimeric protein chains, a "hexamer" will comprise 6 "monomers" for a total of 12 disulphide bonded chimeric protein chains, and a "dodecamer" will comprise 12 "monomers" for a total of 1024 disulphide bonded chimeric protein chains.

"Promoting polymerisation" "Increasing polymerisation"

For the purposes of the present disclosure references to promoting or increasing polymerisation should, except for where the context requires otherwise, be taken as referring to an increase in the size of polymers formed. That is to say, for example, an increase in the proportion of dodecamers formed, as compared to the number of pentamers or hexamers. This increase in the size of polymers formed may optionally occur in combination with an increase in the proportion of the polypeptides being incorporated in polymers.

"Polymeric proteins"

The polymeric proteins of the invention may be provided in the form of any suitable multimer, including, but not limited to, pentamers, hexamers, heptamers, nonomers, decamers, undecamers, and dodecamers. In a suitable embodiment, a polymeric protein of the invention is in the form of a dodecamer.

The invention will now be further described with reference to the following Examples and accompanying figures, in which:

Figure 1. Structural characterization of hexa-Fc
(**A**) Shown are top and side views of the hexamer with the C309 and tailpiece cysteine (here, C366) residues as van der Waals (vdW) spheres. The C309 residues in

neighboring monomers are covalently linked to each other in this model. The tailpiece cysteines in randomly selected monomers are also joined together. (**B**) Tapping mode atomic force microscopy (AFM) images of hexa-Fc show structures of a size and shape consistent with the modeled hexameric complexes. Scale bar: 500nm, main figure; 60nm inset.

Figure 2. Hexa-Fc binds to $CD19^+$ human B cells and $CD14^+$ monocytes

Characteristic FACs plot showing different populations of human peripheral blood mononuclear cells represented by forward (*FSC*) and side (*SSC*) scatter profiles (left panel). (A) Individual $CD19^+$ B cells stained with anti-human CD19-FITC were gated (middle panel). Binding of 50µg of hexa-Fc to gated human $CD19^+$ B lymphocytes was detected using phycoerythrin (PE)-labeled goat (Fab'_2) anti-human IgG (right panel).

Figure 3. Hexa-Fc binds Fc-receptors with high avidity

(A) Hexa-Fc binds to FcRL5 and FcγRIIb (CD32). Binding of either heat-aggregated IgG1 (left panel) or hexa-Fc (right panel) to cells expressing FcRL5, FcγRIIb, CD200R control, or FcRL4 control. Binding to CD200R and FcRL4 (human IgA receptor) are included as two negative controls. Data are representative of duplicate experiments. (B) Improved binding of hexa-Fc when FcγRIIb and FcRL5 are simultaneously expressed on the surface of 293 cells. Binding of hexa-Fc (right panel) to FcRL5/CD32 double transfectants, FcRL5 single transfectants, FcRL4/CD32 double transfectants and CD32 single transfectants. CD200 transfected controls are omitted from the overlays for clarity. Binding of hexa-Fc and heat-aggregated IgG to the FcRL5/CD32 double transfectants is enhanced when compared with FcRL5 single transfectants. In contrast with heat-aggregated IgG (left panel) binding by hexa-Fc was predominantly to FcγRIIb as binding by the FcRL5/CD32 double-transfectants was not much greater than that seen to either FcRL4/CD32 double- or CD32 single-transfectants. Data are representative of duplicate experiments. Cell surface expression of FcRL5, FcRL4 and CD200R were confirmed using FITC-conjugated anti-FLAG M2 mAb or by staining with anti-CD32 Ab.

Figure 4. Hexa-Fc preferentially binds FcγRIIb over FcRL5

FcRL4/CD32 (top panel) or FcRL5/CD32 (bottom panel) double-transfectants were pre-incubated with anti-FcRL5 blocking mAb 509F6 or anti-FcRL4 blocking mAb 413D12. Anti-FcRL5 blocking antibody did not reduce binding of hexa-Fc but markedly reduced binding of heat-aggregated IgG, showing that hexa-Fc prefers to bind FcγRIIb when given

the choice of either receptor. Remaining traces show binding by 509F6 and 413D12 in the absence of human Igs. Cell surface expression of FcRL5 and FcRL4 were confirmed using FITC-conjugated anti-FLAG M2 mAb or by staining with anti-CD32 Ab. Data are representative of duplicate experiments.

Figure 5. Binding of hexa-Fc to human DC-SIGN by multi-channel surface plasmon resonance analysis (SPR). Association and dissociation curves of Igs binding to recombinant human DC-SIGN immobilized on a sensor chip. Hexa-Fc, dimeric-Fc or gp120 control were injected at doubling dilutions as indicated into flow at time 0, and replaced with buffer at 300 sec. Data are representative of duplicate experiments.

Figure 6. Binding of IgM-Fc to human DC-SIGN by multi-channel surface plasmon resonance analysis (SPR).
Association and dissociation curves of IgM-Fc and Pentaglobin™ binding to recombinant human DC-SIGN or SIGNR1 immobilized on a sensor chip. IgM-Fc or Pentaglobin™ were injected at doubling dilutions from 10µM to 0.32µM into flow at time 0, and replaced with buffer at 300 sec. Data are representative of duplicate experiments.

Figure 7. (A) *N*-glycan profile of hexa-Fc and two different IVIg preparations MALDI-TOF mass spectra of permethylated N-glycans of hexa-Fc, dimeric-Fc, GammaGard™ IVIg, and Malawian IVIg were obtained from the 50% MeCN fraction from a C18 Sep-Pak column ("Materials and Methods"). Annotated structures are according to the Consortium for Functional Glycomics guidelines. All molecular ions are $[M+Na]^+$. Putative structures are based on composition, tandem MS/MS, and biosynthetic knowledge. Due to the presence of heterogeneous multiantennary structures with extended LacNAc repeats, the annotations are simplified throughout by using biantennary structures with the extensions listed in parentheses. Structures that show sugars outside a bracket have not been unequivocally defined. Circled in red is the sugar modelled in the MD simulations. (B) MALDI-TOF-TOF fragment ion spectra obtained after collisional activation of the molecular ion at m/z 2244 showed a core fucosylated structure, as fragments at m/z 474 and 1793 were observed. No fragments showing terminal anntenal fucose residues were detected.

Figure 8. Microtiter wells (Nunc) were coated with DC-SIGN at 10µg/ml in carbonate buffer pH9 and incubated over night at 4°C prior to blocking for 2h at room temperature (RT) in TSM (20mM Tris-HCl, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 5% BSA) buffer pH

7.4. The wells were washed four times with TSM before addition of 100µl digested or undigested antibodies at 10µg/ml in TBS buffer to duplicate wells. After overnight incubation at 4°C and washing as above, alkaline phosphatase-conjugated anti-human IgG or IgM (1:1000; Sigma) was added and incubated for 2h at RT. Wells were washed as above, and 100 µl of the substrate p-nitrophenyl phosphate (Sigma) added to each well and the absorbance measured at 405nm. Antibodies were treated with enzymes according to manufacturer's instructions. Errors bars = SD; n = 2.

Figure 9. Hexa-Fc binds C1q and activates the classical pathway
C1q and C5b-9 deposition to antibodies as detected by ELISA. The figure shows the mean of three experiments.

Figure 10. Structure of FcRL5 and Fc/FcRL5 determined from homology modeling and MD simulations
(A) Shown is the structure of the FcRL5 model near the end of equilibration simulations, showing well formed secondary and tertiary structures that are expected from the crystal structure of FcγRI, which was used as the initial template. (B) Overview of the structure of the Fc/FcRL5 complex (A). The known structure of Fc/FcγRIII was used to initially position FcRL5 in contact with the Fc domain. (C) Detailed view of the contact region of the Fc/FcRL5 complex. The Fc residues that are frequently within 3Å of FcRL5 near the end of the equilibration simulation are shown to give a sense of the number and scope of contact region. Although these proteins remained in contact for the duration of the simulations, the contact was weaker than that in the Fc/FcγRIII complex.

Figure 11. Structure of the glycosylated Fc domain determined from MD simulations
Initial structure of the complex. In the bottom view, the atoms of the complex are depicted as van der Waals spheres to more closely reflect the physical structure. The major part of the structure is the hFc, while the the sugars are as depicted in the schematic of the $Man_5GlcNAc_2$ glycan shown on the left, where mannose residues are circles, the N-acetylglucosamines are squares, and the asparagine residue is an oval. Two views of the complex, differing by 90^0 rotation about the long axis, are shown. The accessiblity of the mannose residues for interactions with lectins is limited in this structure owing to the size of the internal Fc cavity and the location of the sugars. (**B**) Structure of the monomer after ~125ns. In this, one glycan chain remains closely associated with the $C_\gamma2$ domain and remains buried within the cavity. However, the other chain has adopted a structure that interacts with the $C_\gamma2$ domain only via the di-N-acetylchitobiose core. In this more loosely

bound configuration, the α1-6 mannose branch residues of the glycan (circled in the schematic in (**A**)) are near to the cavity entrance, more accessible to potential interactions with lectins such as DC-SIGN.

Figure 12. Evaluation of the accessibility of the α1,6 branch mannose residues for the DC-SIGN CRD. The CRD is positioned so that the mannose residues in the crystal structure overlap those in the Fc-glycan. The van der Waals representation of the structure (right) more closely reflects the physical structure of the complex.

Figure 13. N236A and D001N/N236A mutants run at a molecular weight consistent with the formation of dodecamers

Figure 14. N236A mutant binds human monocytes (in particular CD14 low, CD16 high monocytes).

Figure 15 illustrates results from a study of DC-SIGN binding by series 1 mutants.

Figure 16 illustrates results from a study of activation of complement by proteins of the invention, and comparator proteins.

Examples

Example 1

Introduction

Fc-fusion proteins are a well-established class of therapeutics, in fact presently exhibiting the greatest growth rate of all biologics in the United States. Notwithstanding this success though, there is great interest in identifying novel approaches to improve their efficacy and safety while expanding their range of potential clinical applications to other areas such as vaccines and replacements for intravenous immunoglobulin (IVIG) therapy. However, one well-recognized drawback of the present Fc-fusion design for many of its potentially new applications is its monomeric structure: it is not able to cross-link multiple receptors with the high affinity required for enhanced function.

In particular, several diseases are known to be regulated by the activity of low-affinity inhibitory Fc receptors, including those on the surface of human B cells, such as FcγRIIb

and FcRL5 and those on macrophages and dendritic cell (DC) surfaces, such as FcγRIIb and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Of note, DC-SIGN is a C-type lectin and indeed there is a strict requirement of glycosylation for its association with IgG. In particular, a number of studies have implicated □2,6-sialylation of the Fc-glycans as critically important for this interaction with DC-SIGN, although there is recently a great deal of debate on this issue.

Intriguingly, each of these receptors is also targeted by pathogens in their attempt to inhibit immune responses involved in their removal. Taken together, FcγRIIb, FcRL5, and DC-SIGN may thus limit immune cell activation against chronic pathogens or self-reactive antigen, and approaches that have the potential to target these receptors with high affinity/avidity may prove beneficial in therapies, including IVIG, aimed at controlling pro-inflammatory disease.

We also note that the monomeric structure of present Fc-fusions also prevents their interaction with complement, which significantly limits their application in cancer therapies where complement activation may be desirable. Multimerization would also be expected to significantly enhance their interaction with the salvage neonatal Fc-receptor (FcRn), a crucial association that significantly prolongs the plasma half-life and likewise therapeutic and/or vaccine activity of any Fc-containing protein.

We have recently developed an effective strategy to polymerize monomeric Fc into well-defined hexameric polymers (hexa-Fc) and demonstrated their binding to high-affinity Fc receptors. Here we characterize the functional characteristics of this unique biosynthetic nanoparticle with several important immune effector systems, including low affinity B- and dendritic cell (DC) receptors, complement, and FcRn. We show that the binding to these effectors is strong, as expected from its polymeric architecture, and thereby firmly establish this novel Fc nano-scaffold as an extremely promising alternative for future therapeutic and vaccine approaches.

Results

Binding of hexameric IgG1-Fc to human leucocytes

We previously generated hexameric human IgG1-Fc (hexa-Fc) that bound human FcγRI, FcγRIIa^{R131}, FcγRIIb, and mouse FcγRI, FcγRIIb with higher affinity than monomeric or dimeric human IgG1-Fc, as expected from its increased valency (Czajkowsky et al, 2012;

Mekhaiel et al, 2011) (Fig 1). As a first step to evaluate the interaction of hexa-Fc with human immune cells, we determined whether hexa-Fc binds to human circulating B cells and monocytes. To this end, $CD19^{+}$ B cells from peripheral blood mononuclear cells of healthy human volunteers were screened by flow cytometry analysis. Despite high background binding of the anti-human IgG detecting reagent, most likely due to direct interactions with the IgG B cell receptor (BCR) and/or pre-bound IgG found on B cells, we nonetheless detected increased binding of hexa-Fc (Fig 2A). We could also observe a robust association of hexa-Fc to $CD14^{+low}$ and to a lesser extent to $CD14^{+high}$ monocytes from these same individuals (data not shown).

FcRL5 and Fc RIIb are receptors for hexameric IgG1-Fc

Human B cells are known to express two FcγRs for IgG, FcγRIIb (CD32) and FcRL5 (Daeron *et al*, 1995; Franco *et al*, 2013; Pritchard & Smith, 2003; Wilson *et al*, 2012). To determine if these receptors could contribute to the interaction of hexa-Fc with B cells, and to overcome issues of background binding observed with isolated B cells, we evaluated the extent of binding of hexa-Fc to 293 cells transiently expressing these proteins or control CD200R and FcRL4 receptors by FACS (Wilson *et al*, 2010). We also studied the ability of heat-aggregated IgG to bind to these cells as a positive control and to provide some structural insight into the nature of these interactions. We found that both hexa-Fc and heat-aggregated IgG each bound significantly to the FcRL5- or FcγRIIb- expressing cells, whereas no binding was observed to cells expressing either control receptor (Fig 3A). For FcγRIIb, this finding is broadly in line with observations made for IgG1 ICs, which bind appreciably to this receptor (Lux et al, 2013; Schwab et al, 2012). We note that a more pronounced binding of hexa-Fc to FcγRIIb- than FcRL5-expressing cells was consistently observed, while the extent of expression of these receptors was the same.

Binding preferences of FcRL5 and FcγRIIb for hexa-Fc and heat-aggregated IgG

We hypothesized that simultaneous expression of both FcRL5 and FcγRIIb would lead to enhanced binding of heat-aggregated IgG or hexa-Fc. Although a marked improvement in binding of hexa-Fc was observed to the FcRL5/FcγRIIb double transfectants than to cells singly expressing FcRL5, the binding was no more than observed with FcγRIIb single- or FcRL4/FcγRIIb double-transfectants (Fig 3B). This finding suggests that the binding of hexa-Fc to FcγRIIb was preferred over that to FcRL5. In contrast, heat-aggregated IgG bound to the transfectants in a predominantly FcRL5-dominated manner, as the binding to FcRL5/FcγRIIb double transfectants was comparable to that of cells singly expressing FcRL5 and greater than to the FcγRIIb single-transfectants. Further support of these

receptor preferences is evidenced by the blocked binding of heat-aggregated IgG to cells first incubated with the anti-FcRL5 blocking mAb 509F6, whereas binding of hexa-Fc was less affected with this treatment (Fig 4).

Interactions with FcRn

Hexa-Fc was previously shown not to bind human FcRn (Mekhaiel et al, 2011). In contrast to FcγRs, the binding site for FcRn on IgG is localized within the Cγ2-Cγ3 junction and involves residues Ile253, His310, His433 and His435 (Rath et al, 2013; Shields et al, 2001). The pKa of histidine is 6.0-6.5 such that several histidine residues become protonated below physiological pH, allowing for salt bridge formation with acidic residues on the FcRn, thus explaining the strict pH dependency of IgG-FcRn interactions (Raghavan et al, 1995). We reasoned that the lack of binding observed previously with hexa-Fc was due to the presence of leucine at 310 rather than a histidine, as found in IgG1-Fc. At the time we postulated that a histidine residue so close to the critical Cys309 might promote oxidation of the Cys309 and thereby jeopardize polymerization (Salsbury et al, 2008).

We therefore reinserted histidine at 310 to generate hIgG1-Fc-CL309/310CH-TP and investigated the consequence of this mutation on polymerization and binding to FcRn (data not shown). In contrast to the wild-type molecule, hIgG1-Fc-CL309/310CH-TP, bound human FcRn in a pH dependent manner (data not shown), while having no detrimental impact on the ability of these molecules to polymerize into hexamers (data not shown).

Hexa-Fc binds DC-SIGN in a valence dependent manner

To test if hexa-Fc could bind to other, non-classical Fc-receptors that are also believed to be involved in controlling disease, we investigated the interaction of hexa-Fc with soluble recombinant human DC-SIGN tetramers by multichannel surface plasmon resonance analysis (MC-SPR) (Becer et al, 2010; Mitchell et al, 2001; Schwab et al, 2012; Sondermann et al, 2013). Indeed, the sensorgrams show that hexa-Fc binds to DC-SIGN with high affinity (Kd of ~1.48μM) in a dose-dependent fashion (Fig 5). This interaction was stronger than that to dimeric-Fc, likely owing to the greater valency of the hexa-Fc. Interestingly, we also observed that the binding of hexa-Fc to DC-SIGN was stronger than that of IVIg GammaGard® (Fig 5). Finally, we note that we did not detect any significant interactions between hexa-Fc and SIGNR1, the mouse orthologue of the human DC-SIGN, whereas the control HIV gp120, a well-studied DC-SIGN ligand known to carry

substantial amounts of *N*-linked high mannose oligosaccharides, bound to both DC-SIGN and SIGNR1 (Fig 5), as previously reported (Geijtenbeek *et al*, 2000b; Geijtenbeek *et al*, 2000c; Yu *et al,* 2013). The gp120 bound to DC-SIGN and SIGNR1 with high affinity and slow off-rates were observed consistent with the avidity associated with the clustering of carbohydrate-recognition domains within oligomers (Mitchell *et al*, 2001). For the DC-SIGN-gp120 interaction, K_D was determined as 4.39 x 10^{-9} M; k_{on} = 3.6 x 10^4 $M^{-1}s^{-1}$; k_{off} = 1.58 x 10^{-4} s^{-1}. For SIGNR1-gp120 interactions K_D was determined as 3.41 x 10-9 M; k_{on} = 2.77 10^4 $M^{-1}s^{-1}$; k_{off} = 9.46 x 10^{-5} s^{-1}. For hexa-Fc binding to human DC-SIGN, overall affinity was lower when compared with gp120. This is to be expected from the lower density of favoured high-mannose glycans on the Fc polypeptide. However, the off-rate determined was still relatively slow, indicating that once bound, the hexa-Fc-DC-SIGN complex is stable. The K_D was determined as 1.26 x 10^{-6} M; k_{on} = 6.68 x 10^2 $M^{-1}s^{-1}$; k_{off} = 8.39 x 10^{-4} s^{-1}.

IgM-Fc and IVIg enriched for polymeric Igs also bind DC-SIGN

As binding of hexa-Fc to DC-SIGN appeared to be partly owing to its high valency, we hypothesized that DC-SIGN binding may be a shared property of other polymeric antibodies. To explore this possibility, we investigated the binding of a CHO cell derived recombinant (hexameric) IgM-Fc (Mekhaiel et al, 2011) to DC-SIGN and SIGNR1 (Fig 6). The sensorgrams reveal that IgM-Fc binds to DC-SIGN with nanomolar affinity (Kd of ~0.25 μM). Further, in contrast to hexa-Fc, the IgM-Fc also bound strongly to SIGNR1 (Kd of ~2.2 μM). To test if this binding could be recapitulated with native antibodies, we investigated binding of Pentaglobin®, a clinically available IVIg preparation used in the treatment of sepsis and enriched for polymeric Igs (12% IgM, 12% IgA and 76% IgG by weight) (Hoffman *et al*, 2008). In contrast to IgM-Fc, Pentaglobin® bound human DC-SIGN but not SIGNR1 (Fig 6), a finding that may be attributed to differences in glycans or other undetermined posttranslational modifications that arise when expressing proteins in CHO cells.

Hexa-Fc and IVIg exhibit differential glycosylation patterns

Since the interaction of IVIg with FcRL5 (Franco *et al*, 2013), FcγRIIb (Samuelsson *et al*, 2001) and DC-SIGN (Anthony et al, 2011; Schwab et al, 2012; Sondermann et al, 2013) has been attributed to sialylated Fc, we next investigated the nature of the *N*-glycans on hexa-Fc and compared them with two different IVIg preparations (GammaGard™ or Malawian IVIg) and the dimeric-Fc (Fig 7A). MS analysis of hexa-Fc revealed a paucity of sialylated structures but enrichment for high mannose glycans ($Man_5GlcNAc_2$,

$Man_6GlcNAc_2$). This glycan profile is also consistent with observations that DC-SIGN binds high mannose structures (van Liempt *et al*, 2006). MS/MS fragmentation was performed on ions whose masses were consistent with the presence of fucose in order to determine whether hexa-Fc contains terminal antennal fucose residues such as in the Lewis X antigen which can also bind DC-SIGN. These experiments ruled out antennal-linked fucose. For example, MS/MS of m/z 2244 shows a core rather than terminal location for the fucose (Fig. 7B), indicating that the DC-SIGN binding affinity for hexa-Fc is likely the result of increased avidity binding mediated by mannose. The MS analysis also revealed hexa-Fc to be richer in larger multi-antennary and polylac containing N-glycans (for example m/z 2693, 3143 and 3504) which would present more terminal galactose when compared to IVIg N-glycans (Fig 7).

DC-SIGN binding of hexa-Fc and to a lesser extent IgM is critically dependent on the presence of *N*-linked glycans

To confirm that the interaction between DC-SIGN with hexa-Fc and IgM is dependent on the presence of glycans, these carbohydrates were removed from hexa-Fc, IVIg, and human IgM with peptide *N*-glycosidase (PNGase) F, and their resulting ability to bind human DC-SIGN investigated by ELISA (Fig. 8). De-glycosylated hexa-Fc was indeed unable to bind DC-SIGN, demonstrating that binding by hexa-Fc was fully dependent on a PNGase F susceptible glycan(s), such as the aforementioned high mannose residues. By contrast there was ~50% and ~30% residual binding seen with PNGase F treated human IgM and IVIg respectively (Fig. 8). Cleavage of hexa-Fc or IVIg with EndoS or EndoH had little effect on DC-SIGN binding (Fig. 8).

Hexa-Fc binds complement C1q and activates complement via the classical pathway

Binding of C1q and activation of the classical complement pathway was assessed using ELISA. Hexa-Fc bound C1q more efficiently than either IVIg or IgM (Fig. 9A), a finding that was also reflected in their ability to activate complement to its terminal C5b-9 components (Fig. 9B).

Modelling of hexa-Fc binding to inhibitory receptors, FcRL5 and DC-SIGN.

The results described above indicated two observations that, based on previous work, were somewhat unexpected: namely, the binding of FcRL5 to hexa-Fc demonstrated that

this interaction did not require the presence of Fab or $F(ab')_2$ domains and the binding of hexa-Fc to DC-SIGN appeared to be mediated by mannose-containing glycans. We sought structural insight into these observations by extensive all-atom molecular dynamic simulations.

For the former, we first generated a model of human FcRL5 based on its high homology to FcγRI (Davis, 2007), whose structure is known (Lu *et al*, 2011). Following minimization and extended equilbration simulations (>40ns), the structure was found to adopt an architecture of stable secondary and tertiary structure consistent with expectations based on the FcγRI model (Fig 10A). One notable difference though is the relative disposition of the D1 and D2 domains, which exhibits a hinge angle of ~35^0 in the FcγRI crystal structure yet is ~50^0 in this FcRL5 structure. We verified with simulations of a similar duration that the D1-D2 hinge angle in FcγRI maintains a lower value (~30^0) for the duration of the simulations. However as discussed previously (Lu *et al*, 2011), the D1-D2 hinge angles of low affinity FcγRs, FcγRII and FcγRIII, are also much larger than FcγRI (52^0 - 55^0), and such a sharply bent D1/D2 structure might only be a feature of high affinity FcγRs. Hence, a larger D1/D2 hinge angle in the low affinity FcRL5 is consistent with other low affinity FcγRs, and its observation in the equilibrated FcRL5 model here thus provides further support for its accuracy.

We then placed this equilibrated FcRL5 structure in the analogous position of FcγRIII in the known structure of Fc/FcγRIII (Sondermann *et al*, 2000) and performed extensive equilibration simulations (>120ns). For comparison, we also performed similarly long simulations on the Fc/FcγRIII complex. Despite the lack of Fab domains, FcRL5 remained in contact with the Fc domain for the duration of the simulations (Fig 10B and Fig 10C). As with FcγRIII (Sondermann *et al*, 2000), FcRL5 interacted with both Fc heavy chains, one predominantly in the D1/D2 junction and the other within the D2 domain, although the number of these associations were significantly lower than in the FcγRIII/Fc complex. In particular, the heavy chain interaction with the D1/D2 junction in FcRL5 was markedly weaker than in FcγRIII complex. Hence, these findings suggest that indeed FcRL5 can interact with just the Fc domain but this interaction is weaker than that of FcγRIII/Fc, and might thus more strongly depend on oligomerization of Fc domains to enable a sufficiently stable interaction (as seen in Figs 3 and 4).

As for the putative interactions between mannose-glycans and DC-SIGN, as a first step towards a structural understanding of this association, we noted that there were

crystallographic data of a human Fc domain with high mannose glycans (Crispin *et al*, 2009). Using this structure as a template, we constructed a model of the human Fc domain (mutated in two residues to enable polymerization into the hexa-Fc (Mekhaiel et al, 2011)) containing the $Man_5GlcNAc_2$ glycan that MS identified here as attached to hexa-Fc, and evaluated the structure with equilibration MD simulations.

Immediately apparent with this initial structure however was the limited accessibility of the mannose residues for any putative lectin: the entrance to the internal cavity of the Fc domain (where the glycans are located) is roughly elliptical, with dimensions of 2nm x 3.5nm and the mannose residues are deeply buried within this cavity (Fig 11A). With each carbohydrate recognition domain (CRD) of the tetrameric DC-SIGN shaped as a sphere of 3nm diameter (Feinberg *et al*, 2001), this Fc-glycan structure poses significant limitations for potential interactions with DC-SIGN.

However, once equilibrated, the complex frequently adopted a configuration in which the α1-6 branch mannose residues that are expected to interact with DC-SIGN (Feinberg *et al*, 2001) are located near the entrance to the Fc cavity (Fig 11B). During the simulation, both glycan chains essentially adopt one of two configurations: one in which the di-N-acetylchitobiose core, the central β mannose, and α1-6 branch residues are all in close proximity to the $C_\gamma2$ domain (similar to the glycan structures observed in earlier crystallographic studies (Crispin *et al*, 2009; Harris *et al*, 1997; Matsumiya *et al*, 2007)) and a previously uncharacterized configuration in which only the di-N-acetylchitobiose core is close to the $C_\gamma2$ domain. The α1-3 branch mannose residue in both configurations is essentially always oriented towards and frequently interacting with the other glycan chain. While one glycan chain predominantly adopted only the former structure (96.7% of the trajectory, see Methods), the other chain frequently adopted the latter configuration (34.5% of the trajectory), and it is in this latter configuration that the α1-6 branch mannose residues were localized near to the entrance of the cavity. We verified that at this location these mannose residues are indeed accessible to potential interactions with DC-SIGN (Fig 12). We note that this Fc structure can also be assembled into a barrel-shaped hexameric architecture of the hexa-Fc following the structural principles previously described (Mekhaiel et al, 2011) (Fig 1).

Discussion

Previous studies suggested that FcRL5 might be a receptor for IgG (Ehrhardt *et al*, 2003; Haga *et al*, 2007). However, binding of soluble monomeric IgG to FcRL5-transfected 293

cells was not observed in FACS-based assays, indicating that FcRL5 was likely to be a low- to medium-affinity Fc receptor, if at all (Wilson *et al*, 2012).

Our data clearly show that FcRL5 can bind complexed IgG1 and that this interaction can occur in the absence of the Fab or $F(ab')_2$ regions, as hexa-Fc does not contain these domains. MD simulations further show that this interaction can involve similar Fc regions as implicated in the interaction with classic FcγRs (Franco *et al*, 2013; Sondermann *et al*, 2000; Woof & Burton, 2004), although the number and strength of these associations is noticeably lower than at least the FcγRIII/Fc complex. This suggests that the observed significant binding of FcRL5 to hexa-Fc is likely owing to the polymeric nature of the complex, which would explain the failure to observe the aforementioned FACS-based assays with monomeric IgG (Wilson *et al*, 2012).

These findings are in agreement with two recent publications that have studied the interaction of IgG with FcRL5 (Franco *et al*, 2013; Wilson *et al*, 2012). One of these studies (Franco *et al*, 2013) showed that a stronger interaction with monomeric IgG may involve contributions from both the IgG Fc and IgG $F(ab')_2$, unlike classic FcγRs such as FcγRIIb that only bind via the Fc (Woof & Burton, 2004). Our observation of a stronger interaction of hexa-Fc to FcγRIIb than FcRL5 may thus be owing to an inherently stronger interaction with the Fc domain in FcγRIIb compared to FcRL5. The finding that heat-aggregated IgG binds more strongly to FcRL5 than to FcγRIIb, also consistent with previous work (Franco *et al*, 2013; Wilson *et al*, 2012), may thus be owing to the additional contact with $F(ab')_2$ that do not occur in the FcγRIIb interaction.

Our data with hexa-Fc show that reagents can be designed that engage both FcγRIIb and FcRL5 with high affinity in a uniquely Fc valence-dependent manner. Our study also shows that reagents can be designed that bind FcγRIIb preferentially over FcRL5 (Fig 4). Since FcRL5 and FcγRIIb are both expressed by activated B cells, blocking the ability to bind existing Ab with hexa-Fc may potentially augment the Ab titer and repertoire generated by vaccines. Further, the expression of FcRL5 by antigen experienced B cells makes it an attractive target for Ab-mediated cellular depletion (Elkins *et al*, 2012). Autoimmune disorders that are dependent on autoantibodies for their pathology may therefore be successfully controlled by hexa-Fc.

DC-SIGN signalling invokes IL-10 production which is of significance in anti-inflammatory pathways (Gringhuis *et al*, 2009; Gringhuis *et al*, 2007). Recent work showing that DC-

SIGN and SIGNR1 are important receptors in the efficacy of IVIg in controlling autoimmune disease (Anthony et al, 2011; Schwab et al, 2012) prompted us to investigate the interaction of these additional receptors with hexa-Fc (Fig 5). Indeed, we found that hexa-Fc bound more strongly to DC-SIGN than monomeric IgG, and that this interaction was wholly dependent on glycans as their removal with PNGase F resulted in a molecule unable to bind the receptor (Fig. 8).

The interaction of IgG with DC-SIGN has recently been ascribed to terminal sialylation of the N-linked glycan at position 297 in the Fc (Schwab et al, 2012; Sondermann et al, 2013). Contrary to expectations though, we found that the glycans attached to hexa-Fc were generally more diverse, being rich in both terminal mannose and galactose, and that terminal sialylation was rarely observed (Fig 7A). Our hypothesis that mannose but not sialic acid is critical to binding of DC-SIGN by hexa-Fc is supported by the fact that □ 2,3 disialyl linkages applied by CHO (as with hexa-Fc) are apparently not involved in amelioration of autoimmune disease by recombinant Fc (Anthony et al, 2011; Schwab et al, 2012; Schwab & Nimmerjahn, 2013). We also note that the role of sialic acid (but not mannose) in ITP (Leontyev *et al*, 2012) and binding by DC-SIGN or SIGNR1 has been questioned by numerous recent studies (Crispin *et al*, 2013; Leontyev *et al*, 2012; van Liempt *et al*, 2006; Yu *et al*, 2013). Finally, our MD results provide a structural means by which an interaction mediated via mannose residues could occur, which appeared challenging based on available crystallographic data of glycosylated-Fc (Harris *et al*, 1997). We note that this orientation differs from that previously identified by nuclear magnetic resonance (Barb & Prestegard, 2011), as the α1-3 branch in this configuration is still within the cavity (Fig 11,12). Hence, together with this other configuration, which was not observed in our simulations perhaps owing to their shorter duration (~150ns), there appears ample means by which lectins, in particular DC-SIGN, may interact with Fc-glycans.

One of the most studied DC-SIGN interactions is that with the influenza virus trimeric haemagglutinin, which can achieve affinities up to 10^8 M^{-1} compared with around 4 x 10^2 M^{-1} when one or both units are not in a multivalent state (Hillaire *et al*, 2013; Londrigan *et al*, 2011; Mammen *et al*, 1998). Hence, as an increase in avidity can enhance an inherently weak substrate affinity, presenting glycan residues on a hexameric-Fc scaffold would likewise be expected to significantly enhance receptor binding, and thus may have potential for treating autoimmune disease. That this is the case has been shown with nitrophenol-specific Abs recognizing sialic acid coupled to nitrophenol that efficiently

assembled IgM-CD22 complexes on the surface of naive B cells (Adachi *et al*, 2012; O'Reilly *et al*, 2008). Not surprisingly IgM as a decavalent protein scaffold was more effective than either IgA or IgG, a finding supported by our observations that IgM-Fc or IgM-enriched IVIg can also bind DC-SIGN (Fig. 6). Human IgM is known to be heavily mannosylated and these glycans are involved in binding of IgM by mannan binding lectin, a member of the collectin family of proteins, which bind to oligomannose and GlcNAc-terminating structures (Arnold *et al*, 2005). Given the observed high-affinity binding of human IgM and Pentaglobin® to human DC-SIGN and SIGNR1, very small amounts of contaminating polymeric Igs that are infrequently found in commercial IVIg preparations (Wu *et al*, 2013), and frequently found in aged IVIg preparations (Teeling et al, 2001), may explain why some groups observe IVIg binding to DC-SIGN and other groups do not.

The glycan analysis also revealed hexa-Fc to be richer in glycans fully capped with terminal galactose when compared with IVIg (Fig. 7A). Since the anti-inflammatory activity of immune-complexed IgG1 is also mediated by Fc galactosylation (Karsten *et al*, 2012), hexa-Fc may be superior to IVIg at promoting the association of FcRIIb with dectin-1, thereby blocking C5a-dependent inflammation *in vivo*, including peritonitis and skin blistering in experimental epidermolysis bullosa acquisita (EBA) for which IVIg is known effective (Czernik *et al*, 2012). As the study by Karsten *et al* so elegantly demonstrates, the multivalent state of the Fc is fundamental to designing reagents that bind inhibitory Fc- and/or glycan receptors optimally (Karsten *et al*, 2012). Hence, presenting glycan residues on the multimeric cylindrical-Fc barrel or bird-cage structure (Fig. 1) would likewise be expected to significantly enhance receptor binding as we have shown here for hexa-Fc interactions with FcRIIb, FcRL5, and DC-SIGN and thus may have greater potential for inhibiting autoimmune disease when translated to an IVIg biomimetic therapy (previous PCT as reference for support of structure).

Hexa-Fc also binds C1q and leads to C5b-9 deposition when coated down onto ELISA plates (see Fig 9). Currently, the presence of a fusion partner greatly interferes with the ability of hexa-Fc to engage complement and FcγRs (Mekhaiel et al, 2011). The lack of binding to FcγRs and C1q is due to the fusion partner blocking access to the FcγR and C1q binding sites (the amino-terminal region of C2 domain, also the lower hinge region) or to a lack of receptor flexibility when fused in the existing hinge architecture (Oi et al, 1984; Saphire et al, 2002; Sondermann et al, 2000). It has long been considered that the hinge region serves as a spacer and mediates segmental flexibility allowing the fusion partner to assume a variety of orientations in space relative to the Fc (Saphire et al,

2002). Modifications to the existing hinge e.g. use of the extended hinge from human IgG3, may therefore move the fusion partner away from the critical FcγRs and C1q binding sites and thereby reinstate effector functions to hexameric Fc-fusions that are critical for clearance.

The inhibitory properties of IVIg are known to result from its ability to scavenge active complement proteins, and studies have shown that high levels of IgG can inhibit the uptake of C3b and C4b onto the surface of sensitized guinea pig and human erythrocytes and prevent complement-mediated tissue damage (Basta et al, 1989; Basta et al, 2003). It is thought that IVIg binds the activated complement components C3b and C4b and therefore prevents their deposition onto target surfaces. A disease model in which IVIg convincingly modulates complement is dermatomyositis (Orange et al, 2006). Dermatomyositis is an inflammatory myopathy mediated by the deposition of complement and formation of the C5-9 membrane attack complex (MAC) on intramuscular capillaries, leading to loss of capillaries and muscle ischemia and necrosis. By acting as a scavenger of complement, hexa-Fc may therefore be a useful therapy for dermatomyositis. However, for diseases where complement activation is neither desirable nor safe, mutations that disrupt C1q binding e.g. K322A, P329A, P331A may be introduced into the wild-type molecules described herein.

A critical feature to the utility of oligomeric Fc-fusion proteins in future drugs or vaccines will be their ability to interact with the FcRn. Here we have shown that His310 is critical to binding of hexa-Fc to human FcRn, although whether this reinstates binding in the context of N-terminal fusions remains to be tested. The nature of the fused partner may potentially affect FcRn binding and interactions with FcRn will therefore most likely need to be determined for each unique fusion. The introduction into hexa-Fc of additional mutations known to enhance interactions of the Fc with FcRn may further enhance (Dall'Acqua et al, 2002; Hinton et al, 2006; Petkova et al, 2006; Vaccaro et al, 2006; Zalevsky et al, 2010) its translational potential. Hexa-Fc now provides a template molecule to further engineer selective gain-of and/or loss-of function mutations, as demonstrated here for FcRn, that allow the exisiting multimeric scaffold to be tailored for optimal use in novel drugs and vaccines.

Materials and Methods

Construction of the CL309/310CH mutant

The CL309/310CH mutant was constructed by PCR overlap extension mutagenesis from the wild-type vector (pFUSE-hIgG1-Fc-TP-LH309/310CL) as the template, using the internal mismatched primer mut-3:5'-ACCGTCTGC**CAC**CAGGACTGG-3' and its complement to incorporate a CTC to CAC substitution and Fcmut-1:5'-ACCCTGCTTGCTCAACTCT-3' and Fcmut-1:3'-TTGATGAGTTTGGACAAACCA-5' as flanking primers. PCR products were then digested using *EcoR*I and *Nhe*I (New England Biolabs) and cloned back into the wild-type vector to generate pFUSE-hIgG1-Fc-TP-CL309/310CH. To verify incorporation of the desired mutation and to check for PCR-induced errors, the entire coding sequence of the new expression plasmid was sequenced on both strands.

CHO-K1 cells (European Collection of Cell Cultures) were transfected with plasmid using FuGene (Promega) and positive clones selected. Cells were grown in DMEM complete media supplemented with 10% ultra-low bovine IgG FCS, 100 IU/ml penicillin, and 100 μgml^{-1} streptomycin (PAA) at 37°C/5%CO2. Stable transfectants were selected in medium containing 400 μgml^{-1} of Zeocin (Invivogen). Clones secreting hexa-Fc proteins were detected by sandwich enzyme-linked immunosorbent assay (ELISA) using goat anti-human IgG (Invitrogen) to capture and goat anti-human IgG-Fc (Sigma-Aldrich: A0170) conjugated to horseradish peroxidase to detect. The CL309/310CH mutant was purified from larger scale cultures on Protein-G-Sepharose (GE Healthcare, Little Chalfont, Bucks, UK) using an AKTA FPLC (GE Healthcare).

Ig binding assays

The generation of hexa-Fc has been previously described (Mekhaiel et al, 2011). Peripheral blood mononuclear cells (PBMC) were purified from buffy coats kindly provided by human volunteers using Lymphoprep™ (Axis-Shield) according to manufacturer's instructions. All work was conducted after approval by the Ethical Review Committee of the Liverpool School of Tropical Medicine.

1×10^5 PBMCs were incubated with 200µl FACs buffer (phosphate- buffered saline, 0.2% bovine serum albumin, 5% goat serum) containing 50µg of hexa-Fc or buffer only as indicated for 1h at room temperature. Cells were washed twice with FACs buffer and incubated for 1h at 4°C with 1/500 dilution of $F(ab')_2$ goat anti-hIgG-Fc-phycoerythrin (PE), goat anti-hCD19-fluorescein isothiocyanate (FITC)-conjugated (BioLegend) and goat anti-hCD14-APC-Cy7 (BioLegend) in 200µl FACs buffer. After washing with FACs buffer, cells were analyzed on a FACScan (BD Biosciences). Data acquisition was conducted with

CELLQuest software (BD Biosciences) and the analysis performed with FlowJo version 9.1.

To test for hexa-Fc binding by FcRL family members, cDNA encoding human CD200R, FcRL4, or FcRL5 were ligated into pFLAG-CMV-3 (Sigma). cDNA encoding human CD32 was ligated into pEF6 (Invitrogen) (Wilson *et al*, 2012). Proteins were expressed in 293 cells by transient transfection using Lipofectamine 2000 (Wilson *et al*, 2012). Transiently transfected 293 cells were used for Ig binding assays 36-42 h after transfection. Purified human IgG1 was obtained from Sigma-Aldrich and hexa-Fc was purified as previously described (Mekhaiel et al, 2011). For the heat aggregation assay Igs were aggregated by heating to 60°C for 30 min. Igs were then diluted to 100µg/ml in PBS/1% BSA. The 293 cells were incubated for 30 min on ice with the Igs and washed four times, followed by incubation with biotin-conjugated goat $F(ab')_2$ anti-human IgG (Southern Biotechnology) for 20 min on ice. Cells were washed three times and incubated with FITC-conjugated anti-Flag Ab (M2; Sigma-Aldich) for 20 min on ice. To detect CD32-expressing cells, a PE-conjugated anti-human CD32 Ab (Beckman Coulter) was added to CD32-transfected samples. Cells were washed twice and analyzed by flow cytometry on a FACSCalibur (BD Biosciences) for Ab binding. Dead cells were excluded by propidium iodide staining (Wilson *et al*, 2012).

Recombinant human DC-SIGN was generated as described previously (Mitchell *et al*, 2001). Recombinant SIGNR1 was from R and D systems. Purified recombinant HIV gp120 was a kind gift of Dr Max Crispin (University of Oxford). Soluble recombinant DC-SIGN and SIGNR1 proteins were captured on GLM sensor chips (Bio-Rad laboratories) via amine coupling with sulfo-N-hydroxysuccinimide/1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide and all sensorgrams using soluble-phase analytes of immunoglobulin preparations were recorded at 25°C with the ProteOn XPR36 surface plasmon resonance biosensor (Bio-Rad laboratories) at a flow rate of 25 □ l per minute. Kinetic parameters for protein-protein interactions were determined using the 1:1 Langmuir modeling algorithms included in the ProteOn Manager software suite (Bio-Rad Laboratories). GammaGard™ and Pentaglobin™ were kindly provided by Baxter Healthcare and Biotest UK respectively. The generation of IgM-Fc has been described previously (Mekhaiel et al, 2011). Human serum IgM and IgG (Sigma Aldrich).

Complement binding assays

Antibodies were coated down to 96 well ELISA plates (Nunc) in carbonate buffer pH9 (Sigma) at the indicated concentrations overnight at 4°C. Plates were then washed five times in PBS/0.1% Tween-20 (PBST) before adding normal human serum (NHS) at 1:100 in Veronal buffered saline containing 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Tween-20, 0.1% gelatin and 0.5% BSA and incubated for 2h at room temperature as described previously (Lewis et al, 2008). After washing as above, plates were incubated with a 1:500 dilution of mouse anti-human C5b-9 (Serotec) or peroxidase labeled sheep anti-human C1q (Serotec) in PBST/0.1% BSA for 1h at room temperature. After further washes as above, plates were incubated in anti-mouse IgG (Pierce) diluted 1:500 in PBST/0.1% BSA for 1h prior to washing and developing with p-nitrophenyl phosphate substrate (Sigma).

Human FcRn binding assays

Microtiter wells (Nunc) were coated with titrated amounts of the Fc-fusions (20.0- 0.1 µg/ml) in PBS and incubated over night at 4°C prior to blocking with 4% skimmed milk (Acumedia) for 1 h at room temperature (RT). The wells were washed four times with PBS/0.005% Tween 20 (PBS/T) pH 6.0 before addition of GST-tagged hFcRn in 4% skimmed milk PBS/T pH 6.0 and added to the wells (Andersen et al, 2008). After incubation for 2 h at RT and washing as above, an HRP-conjugated polyclonal anti-GST from goat (1:8000; GE Healthcare) was added and incubated for 1 h at RT. Wells were washed as above before 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate (Calbiochem) was added to each well and incubated for 45 minutes before 100 µl of 0.25 M HCl was added. The absorbance was measured at 450 nm using a Sunrise TECAN spectrophotometer (TECAN, Maennedorf, Switzerland).

N-glycomic analysis

N-glycomic analysis was performed according to a protocol described previously (North *et al*, 2010). Briefly, 50 □ g of each sample was reduced by dithiothreitol (Sigma, Aldrich) and then carboxymethylated by iodoacetic acid (Sigma Aldrich). Samples were subsequently dialyzed, freeze-dried and digested by trypsin (Sigma Aldrich). The peptides/glycopeptides were purified using Oasis HLB Plus Short cartridges (Waters). N-glycans were released from glycopeptides by PNGaseF (Roche Applied Science) and isolated from peptides using Sep-Pak C18 catridges (Waters). The released N-glycans were permethylated, purified by Sep-Pak C18 cartridges again, freeze-dried and dissolved in 10□ l methanol. 1□ l of dissolved sample was premixed with µl of matrix (for MS, 20 mg/ml 2,5-dihydroxybenzoic acid in 70% (v/v) aqueous methanol; for MS/MS, 20 mg/ml 3,4-diaminobenzophenone in 75% (v/v) aqueous acetonitrile). MALDI-TOF MS analysis

using a Voyager-DETM STR mass spectrometer (Applied Biosystems). The data were analyzed using Data Explorer (Applied Biosystems) and Glycoworkbench (Ceroni *et al*, 2008).

Modelling hexa-Fc interactions with FcRL5 and DC-SIGN

The homology model of FcRL5 was constructed with the automated homology modeling tools in DeepView (Guex & Peitsch, 1997), using the human FcRL5 (accession no. Q96RD9) and the crystal structure of the FcγRI (PDB accession codes: 3RJD). The structure (and all models here) was then solvated in TIP3 water (Jorgensen *et al*, 1983) and then minimized and equilibrated using VMD/NAMD (Phillips *et al*, 2005) and the CHARMM36 force field (Best *et al*, 2012), in the constant pressure and constant temperature (NPT, 295K, 1atm) ensemble. The temperature and pressure were controlled by the Berendsen thermostat and barostate with a coupling time of 0.1ps and 1.0ps, respectively. The particle mesh Ewald algorithm was employed to treat electrostatic interactions. The van der Waals interactions were treated with a cut-off of 12Å, and the integration step was set to 2fs. After ~10ns, the protein attained an equilibrated conformation, as judged by the root-mean-square deviation of the protein backbone. The protein secondary and tertiary structures were evaluated with VMD. A similar procedure was followed for the simulations of FcγRI. The D1-D2 hinge angle was determined by measuring the angle subtended by residues Val81, Ala88, and Ala94 in FcRL5 and Ile96, Gly103, and Ser110 in FcγRI. For the model with Fc, the crystal structure of the FcγRIII/Fc (PDB accession codes: 1E4K) was used as a template. The equilibrated structure of FcRL5 was superimposed on the FcγRIII structure, aligning the D1 and D2 domains of FcRL5 with the corresponding domains in FcγRIII, using the least-squares fitting procedure in DeepView. The Fc domain used in these simulations was the human Fc structure of the FcγRIII/Fc complex. For the simulations of the mannosylated IgG, the crystal structure of the human IgG1 (PDB accession codes: 2WAH) was used as the template and initial structure for the model, as it contained high mannose glycans. However, since the glycans present in this structure ($Man_9GlcNAc_2$) were not found to be attached to hexa-Fc by MS, we manually removed the appropriate mannose residues to obtain the initial structure of the $Man_5GlcNAc_2$ glycan, which is attached to the hexa-Fc as shown here by MS (circled in Fig 7). This glycan structure was then attached to both heavy chains of the Fc domain. Finally, the hinge and two residue mutations that enable polymerization into the hexa-Fc (Mekhaiel et al, 2011) were generated. The initial files for the simulation were obtained with Glycan Reader (Jo *et al*, 2011). The simulations ran for ~150ns, and the trajectory was analyzed after the protein had equilibrated after the first

30ns. To distinguish between the two configurations described in the text, the number of $C_\gamma 2$ domain residues with 3Å (roughly, hydrogen-bonding distance) of glycan residues 2 through 6 (see Fig 8A) was calculated throughout the trajectory. Those structures with 2 or fewer residues within this distance were considered as the configuration more loosely associated with the $C_\gamma 2$ domain.

Production of mutants

The generation of hexa-Fc has been previously described in the inventors' earlier patent applications. Each mutant was constructed by PCR overlap extension mutagenesis from the wild-type vector (pFUSE-hIgG1-Fc-TP-LH309/310CL) as the template, using the internal mismatched primers described in Table 2 below and Fcmut1-F: ACCCTGCTTGCTCAACTCT and Fcmut1-R: TGGTTTGTCCAAACTCATCAA as flanking primers. To generate the double-knockouts, N007A was used as the template for N236A, giving N077A/N236A. The same approach was used to create the D001N knockouts on the D001 template. PCR products were then digested using *EcoR*I and *Nhe*I (New England Biolabs) and cloned back into the wild-type vector to generate each mutant. To verify incorporation of the desired mutation and to check for PCR-induced errors, the entire coding sequence of the new expression plasmid was obtained on both strands. CHO-K1 cells (European Collection of Cell Cultures) were transfected with plasmid using FuGene (Promega) and positive clones selected by ELISA, expanded and purified as previously described for hexa-Fc.

Example 2

An important feature of hexa-Fc functionality is the NXS/T N-linked glycosylation site, which gives the recombinant protein an almost mandatory glycosylation of the asparagine residues from the NXS/T site. Glycosylation is important in increasing solubility and in influencing interactions with both glycan and Fc-receptors.

The following study used alterations in the number of glycosylation sites in the amino acid sequences of IgG-IgM fusion proteins to produce proteins with modified glycosylation profiles, which in turn lead to increased protein polymerisation.

A fair assumption is that these glycosylation sites are occupied by oligosaccharide and that one must determine the structure of the glycan. However, in proteins with more than one N-linked glycosylation site, one needs to carefully consider the three-dimensional structure of the protein, as it is not always obvious or predictable which will be occupied

with oligosaccharide and which are not. In the case of the polymeric proteins of the invention discussed herein, the inventors confirmed the occupancy of each of the glycosylation sites by observing a change in the proteins' molecular weight when run under reducing conditions into a 4-12% SDS acrylamide gradient gel (Fig. 13)

Hexa-Fc (SEQ ID NO:1) contains two N-linked glycan attachment sites found at Asn77 (equivalent to Asn297 in the Fc of IgG1) and Asn236 in the tailpiece (equivalent to Asn563 of the IgM tailpiece). To define the potential role of these glycans in mediating the binding of hexa-Fc to DC-SIGN, these carbohydrates were removed from hexa-Fc with peptide N-glycosidase (PNGase) F, and their resulting ability to bind human DC-SIGN investigated by ELISA (Fig. 8). Deglycosylated hexa-Fc was unable to bind DC-SIGN demonstrating that binding by hexa-Fc was fully mediated by a PNGase F susceptible glycan. Thus demonstrating the critical importance of glycans to interactions with receptors.

The unique structure of hexa-Fc therefore imposes significant alterations to both the composition and spacio-functional structure of glycans attached to Asn77 and Asn236 that could not have been predicted from prior art based on the glycan compositions of native IgG, or monomeric Fcs (see Example 1). These unique glycan structures in hexa-Fc will by definition alter the function of the molecule in novel and unique ways.

Given the unanticipated effects of glycans when attached in the hexameric barrel structure we modified the existing scaffold to generate new hexa-Fc glycan variants with novel functions (described in detail in Fig. 15). For example, where interactions with glycan receptors need to be reduced additional N-linked sites may be added (e.g. D001N below). Where interactions with glycan receptors are not required they may be removed individually e.g. N077A without overtly compromising the polymeric structure. Where interactions with glycan receptors are desirable, they can be removed from the tail e.g. N236A while maintaining interactions with DC-SIGN and improving the valency of the molecule from 6 to 12. Or they may be modified by expression in CHO cell variants (as in lec series of CHO cells) e.g. when using Fc-fusions as antigen delivery vehicles where you do not wish to drive tolerance or inhibitory responses via DC-SIGN, FcRL5 or FcgRIIb.

These mutants have been generated on the hexa-Fc template (SEQ ID NO:1) and show unexpected properties that could not have been anticipated from the prior art (discussed

above and below). The same panel of glycan mutants are also being introduced into the Fc of IgG2, IgG3 and IgG4.

We are now taking these next-generation iterants of HexaGard further down the translational pipeline. The mutants in Fig. 15 can readily be produced by CHO-K1 cells. Typical yields for these mutants determined by sandwich ELISAs against titrations of IVIG are wild-type hexa-Fc = 2.87µg/ml; D001N = 9.2 µg/ml; N77A = 0.87 µg/ml; N236A = 1.57 µg/ml; N77A/N236A = 0.72 µg/ml; D001N/N236A = 0.63 µg/ml; D001N/N77A = 0.25 µg/ml; D001N/N77A/N236A = 2.89 µ g/ml.

We show for the first time that it is possible to add N-linked glycans to the hinge of IgG1-Fc to generate a molecule (D001N) that is still capable of polymerizing into hexamers (Fig. 13). This was unanticipated, as N-linked glycans are not typically attached to the hinges of native IgG molecules (or other classes of antibody), as they are presumed to interfere with disulphide bond formation and the capacity of the hinge to therefore act as a flexible linker. Native antibodies e.g. IgA tend to O-glycosylate their hinges for this reason. D001N (residue 221 in IgG and in Fig. 5) consequently migrates at a larger size on gels (Fig. 13).

The D001N variant binds more poorly to DC-SIGN or other glycan receptors.

Since removal of the tailpiece glycan (Asn563) in IgM has been shown to enhance polymer formation (mostly an increase in hexamers over pentamers)(de Lalla et al, 1998), we reasoned that a similar mutation introduced into the hexa-Fc tailpiece (N236A) would also lead to enhanced hexamer formation (Fig. 16 & Table 1). To our surprise >95% of N236A (SEQ ID NO:3) was secreted as a discrete dodecameric species (~ 650 kDa) and no hexamers, pentamers or tetramers were observed as seen with hexa-Fc and that are still clearly observable with the IgM 563 mutant made by de Lalla et al 1998. Mutants N236A (SEQ ID NO:3) and D001/N236A (SEQ ID NO:7) in which the glycan residue in the carboxy-terminal tailpiece were removed run with molecular weights approaching ~650 kDa and ~700 kDa respectively (red arrow in Fig. 16 and confirmed by SEC). The inventors believe that the molecules of this weight are dodecamers of Fc. The N236A mutant (SEQ ID NO:3) bound strongly to human monocytes (in particular CD14 low, CD16 high) and to CD19+ B cells (Fig. 14), and the removal of this glycan did not adversely affect binding to DC-SIGN. As with monomeric IgG, removal of N77 significantly reduced binding to human immune cells and DC-SIGN (Fig. 17), although this mutant was still

capable of forming hexamers (e.g. mutants N077A (SEQ ID NO:2) and D001/N077A (SEQ ID NO:6) in Fig. 16).

In contrast to the IgG1-Fc, formation of dodecameric IgM, dodecameric IgGs, and/or IgM-Fc is likely not possible given additional constraints imposed by the size of the Fc (extra Cγ2 domain in the Fc) and associated $F(ab)_2$ arms in each monomer of Ig. The lack of bulky glycans in the tailpiece, the absence of both Fab domains and the extra constant domain in the Fc, therefore allows more of the unstructured tailpieces in N236A (SEQ ID NO:) to form inter-monomeric disulphide bonds via Cys89 thus allowing for the optimal formation of dodecamers over other polymeric species described in the prior art. Furthermore, the addition of a N-terminal glycan into this molecule (D001N/N236A) did not adversely hinder the formation of these dodecamers and led to decreasedbinding to DC-SIGN. IgM differs significantly from hexa-Fc not only with respect to the number and type of N-linked glycans in the Fc but also in that the Cγ2 domain replaces the hinge region. Therefore mutations derived from work on IgM or IgM-Fc don't necessarily translate to either structural or functional equivalence in the N236A, D001/N236A mutants (SEQ ID NO:3 and SEQ ID NO:7 respectively) or hexa-Fc. Mutants N236A (SEQ ID NO:3) and D001/N263A (SEQ ID NO:7) bound human immune cells as well as wild-type hexa-Fc, indicating that these mutant forms of SEQ ID NO:1 will have beneficial utility in various therapeutic applications where enhanced valency is also required.

By running these proteins under reducing conditions we were able to determine the relative sizes of the various glycans attached to hexa-Fc, such that N001 was larger than N236, which in turn was larger than N77. Larger complex glycans attached to N001 are therefore predicted to contain more complex-type glycans and/or sialic acid residues than the smaller glycans attached to N77 and N236. Furthermore the lack of binding to DC-SIGN by the D001/N77A mutant but not the D001N molecule (Table 1) suggests that the oligomannose type glycans are attached to N77 and therefore molecules that can still polymerize into hexamers (Table 1) yet not bind mannose receptors may be preferred for certain embodiments where interactions with these receptors are not desirable e.g. for biomimetics of IVIG to treat autoimmune disease (and as discussed above)..

We now aim to determine the exact glycan composition of the carbohydrates attached at each of these positions since different glycoforms contribute to binding different Fc and glycan-receptors with differing strengths. For example, Ab glycosylation influences Ab half-life and interaction with cellular immune receptors, as shown here for glycan variants

of hexa-Fc. Such engineered glyco-variants of hexa-Fc are therefore predicted to lead to enhanced *in vivo* properties when used in therapy, as is already known for glyco-engineered mAbs.

The inventors are currently working with all of these molecules in various screens of function and safety to determine which ones will make suitable next-generation replacements for Hexa-Fc.

The prototype molecule Hexa-Fc has already been shown to be effective at treating idiopathic thrombocytopenic purpura (ITP) in mice. No obvious adverse reactions are observed in these studies when this polymeric protein is administered by the i.p. route. The inventors are further testing the properties of hexa-Fc and proteins of the invention in models of dermatomyositis, bullous pemphigoid, and chronic inflammatory demyelinating polyneuropathy in the expectation that they will prove therapeutically useful in the treatment of these conditions.

The key finding of this study is that proteins of the invention have a propensity to polymerise to a greater extent than would be expected from the prior art. Indeed, certain proteins (particularly those comprising SEQ ID NO:3 and SEQ ID NO:7) allow formation of dodecamers with enhanced glycan attachments at the N-terminus that may allow for improved binding to DC-SIGN when expressed as dodecamers rather than hexamers.

These proteins of the invention exhibit properties that indicate their utility in the following applications:

- Improved replacements for IVIG (increased avidity binding to inhibitory glycan- and Fc-receptors involved in controlling pro-inflammatory responses).

- Improved Fc-fusion medicines (by allowing the addition of 24 ligand binding modules versus 12 in hexa-Fc). As disclosed in 1E.

- Improved number of attachment sites for the addition of drugs, toxins and/or other cleavable payloads.

- Improved vaccines by increasing the number of antigens delivered to DCs (the depot effect of adjuvants). The superior binding of N236A to monocytes is therefore highly attractive with respect to vaccines.

Table 1. Effect of glycan mutations on polymerisation

MUTANT	POLYMERIC STATE
Wild-type (HexaGard®)	Hexamers > tetramers >> monomers
N77A	Hexamers > tetramers >> monomers
N236A	Dodecamers >>> monomers
N77A/N236A	Monomers
D001N	Hexamers > tetramers >> monomers
D001N/N77A	Hexamers > tetramers >> monomers
D001N/N236A	Dodecamers > monomers
D001N/N77A/N236A	Monomers

Table 2

Mutant	Primer pairs
N077A	Fcmut5-F: GAGCAGTACGCCAGCACGTAC Fcmut5-R: CTCGTCATGCGGTCGTGCATG
N236A	Fcmut6-F: CCCTGTACGCCGTGTCCCTG Fcmut6-R: GGGACATGCGGCACAGGGAC
D001N	Fcmut2-F: GTTAGATCTAACAAAACTCAC Fcmut2-R: GTGAGTTTTGTTAGATCTAAC
LH309/310CH	Fcmut3-F: ACCGTCTGCCACCAGGACTGG Fcmut3-R: CCAGTCCTGGTGGCAGACGGT

Table 3

SEQ ID NO.	Also referred to as	Changes compared to SEQ ID NO:1
1	Hexa-Fc or HexaGard	None
2	N077A	Asparagine at residue 77 substituted with alanine
3	N236A	Asparagine at residue 236 substituted with alanine
4	N077A/N236A	Asparagine at residue 77 substituted with alanine; and asparagine at residue 236 substituted with alanine
5	D001N	Aspartic acid at residue 1 substituted with asparagine
6	D001N/N077A	Aspartic acid at residue 1 substituted with asparagine; and asparagine at residue 77 substituted with alanine
7	D001N/N236A	Aspartic acid at residue 1 substituted with asparagine; and asparagine at residue 236 substituted with alanine
8	D001N/N077A/N236A	Aspartic acid at residue 1 substituted with asparagine; asparagine at residue 77 substituted with alanine; and asparagine at residue 236 substituted with alanine

Sequence information

SEQ ID NO:1 – Hexa-Fc or HexaGard

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYNSTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYNVSLV

MSDTAGTCY

SEQ ID NO:2 – N077A

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYASTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYNVSLV

MSDTAGTCY

SEQ ID NO:3 – N236A

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYNSTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYAVSLV

MSDTAGTCY

SEQ ID NO:4 – N077A/N236A

DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYASTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYAVSLV

MSDTAGTCY

SEQ ID NO:5 – D001N

NKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYNSTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYNVSLV

MSDTAGTCY

SEQ ID NO:6 – D001N/N077A

NKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYASTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYNVSLV

MSDTAGTCY

SEQ ID NO:7 – D001N/N236A

NKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYNSTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYAVSLV

MSDTAGTCY

SEQ ID NO:8 – D001N/N077A/N236A

NKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

GVEVHNAKTK PREEQYASTY RVVSVLTVCL QDWLNGKEYK CKVSNKALPA PIEKTISKAK

GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGKLVL GPPLYAVSLV

MSDTAGTCY

Claims

1. A polymeric protein comprising two or more polypeptide monomer units, each monomer unit comprising two chimeric protein chains;
wherein each chimeric polypeptide monomer unit comprises an Fc receptor binding portion comprising two immunoglobulin G heavy chain constant regions;
wherein each immunoglobulin G heavy chain constant region comprises a cysteine residue which is linked via a disulfide bond to a cysteine residue of an immunoglobulin G heavy chain constant region of an adjacent polypeptide monomer unit; and
and each chimeric protein chain also comprises a modified immunoglobulin M tailpiece region;
wherein the amino acid sequence of each chimeric polypeptide monomer comprises an alteration of the primary structure as compared to the native sequences from which the immunoglobulin G heavy chain constant region or immunoglobulin M tailpiece region are derived, and the alteration changes the number of glycosylation sites in a manner that promotes polymerisation.

2. A polymeric protein according to claim 1, wherein the immunoglobulin G heavy chain constant regions are derived from an immunoglobulin selected from the group consisting of: IgG1; IgG2; IgG3; and IgG4.

3. A polymeric protein according to claim 1, wherein the immunoglobulin G heavy chain constant regions are derived from IgG1.

4. A polymeric protein according to claim 2 or claim 3, wherein the alteration comprises disruption of the glycosylation site corresponding to Asn563 of IgM, or disruption of the glycosylation site corresponding to Asn297 in the Fc of IgG1.

5. A polymeric protein according to claim 4, wherein the alteration comprises disruption of the glycosylation site corresponding to Asn236 of SEQ ID NO:1 or disruption of the glycosylation site corresponding to Asn77 of SEQ ID NO:1.

6. A polymeric protein according to claim 4 or claim 5, wherein the alteration of the primary structure comprises an alteration at a residue corresponding to Asn563 of IgM.

7. A polymeric protein according to claim 6, wherein the alteration of the primary structure comprises an alteration at a residue corresponding to Asn236 of SEQ ID NO:1.

8. A polymeric protein according to claim 6 or claim 7, comprising a sequence selected from the group consisting of: SEQ ID NO:3; and SEQ ID NO:7.

9. A polymeric protein according to claim 8, comprising SEQ ID NO:3.

10. A polymeric protein according to claim 9, consisting of SEQ ID NO:3.

11. A polymeric protein according to claim 8, comprising SEQ ID NO:7.

12. A polymeric protein according to claim 11, consisting of SEQ ID NO:7.

13. A polymeric protein according to claim 4 or claim 5, wherein the alteration of the primary structure comprises an alteration at a residue corresponding to Asn297 in the Fc of IgG1.

14. A polymeric protein according to claim 13, wherein the alteration of the primary structure comprises an alteration at a residue corresponding to Asn77 of SEQ ID NO:1.

15. A polymeric protein according to claim 13 or claim 14, comprising a sequence selected from the group consisting of: SEQ ID NO:2; and SEQ ID NO:6.

16. A polymeric protein according to claim 15, comprising SEQ ID NO:2.

17. A polymeric protein according to claim 16, consisting of SEQ ID NO:2.

18. A polymeric protein according to claim 15, comprising SEQ ID NO:6.

19. A polymeric protein according to claim 18, consisting of SEQ ID NO:6.

20. A polymeric protein according to any preceding claim, wherein the alteration comprises the addition of a further glycosylation site not found in the native sequences from which the immunoglobulin G heavy chain constant region or immunoglobulin M tailpiece region are derived.

21. A polymeric protein according to claim 20, wherein the alteration comprises the addition of a glycosylation site in the hinge region of the immunoglobulin G heavy chain constant region.

22. A polymeric protein according to claim 20 or 21, wherein the addition of the further glycosylation site is at the N-terminal of the immunoglobulin G heavy chain constant region.

23. A polymeric protein according to any of claims 20 to 22, wherein the addition of the further glycosylation site is at position 1 of SEQ ID NO:1.

24. A polymeric protein according to any of claims 20 to 23, comprising a sequence selected from the group consisting of: SEQ ID NO:6; and SEQ ID NO:7.

25. A polymeric protein according to any of claims 20 to 23, comprising the sequence of SEQ ID NO:5.

26. A polymeric protein according to any of claim 25, consisting of the sequence of SEQ ID NO:5.

27. A polymeric protein according to any of claims 1 to 26 for use as a medicament.

28. A polymeric protein according to any of claims 1 to 26 for use as a medicament in the prevention or treatment of an autoimmune or inflammatory disease.

29. A pharmaceutical composition comprising a polymeric polypeptide according to any of claims 1 to 26 and a pharmaceutically acceptable carrier.

30. A method of preventing or treating an autoimmune or inflammatory disease, the method comprising providing a subject in need of such treatment with a therapeutically effective amount of a polymeric protein according to any of claims 1 to 26.

31. A method of intravenous immunoglobulin (IVIG) therapy, the method comprising providing a subject in need of such treatment with a therapeutically effective amount of a polymeric protein according to any of claims 1 to 26.

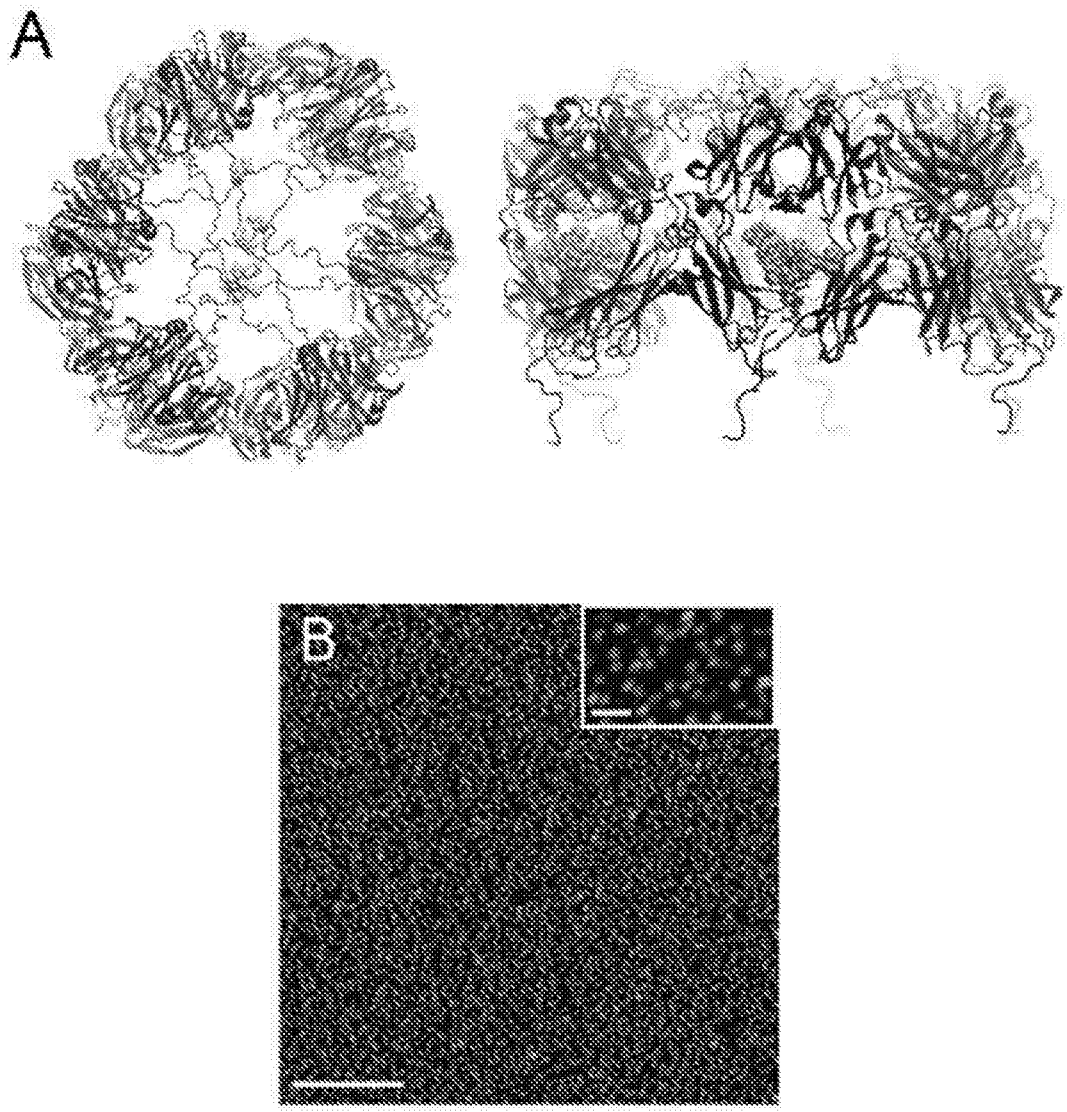

Figure 1

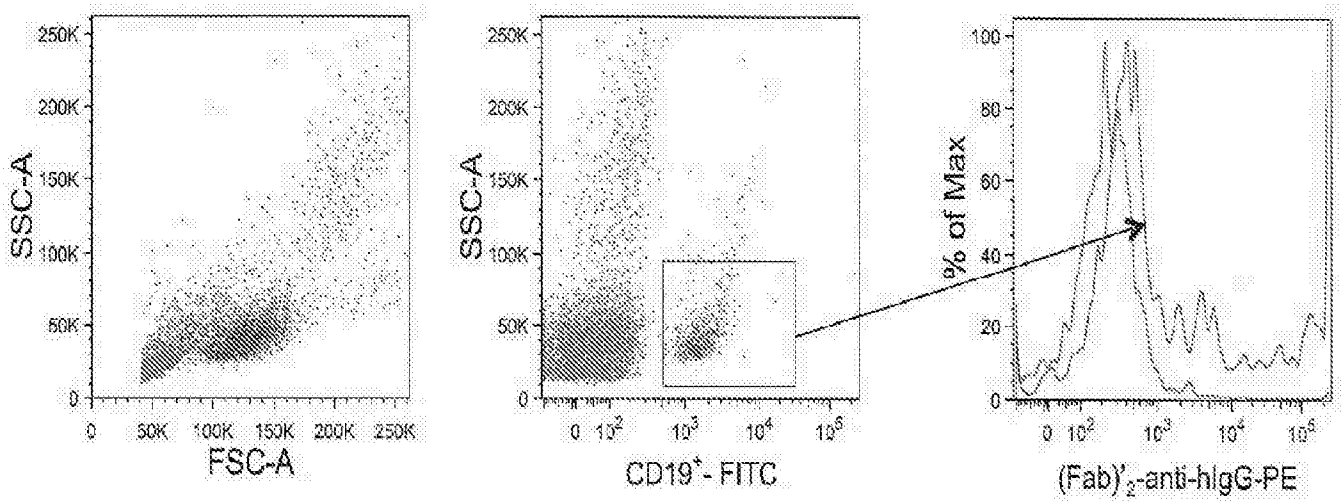

Figure 2

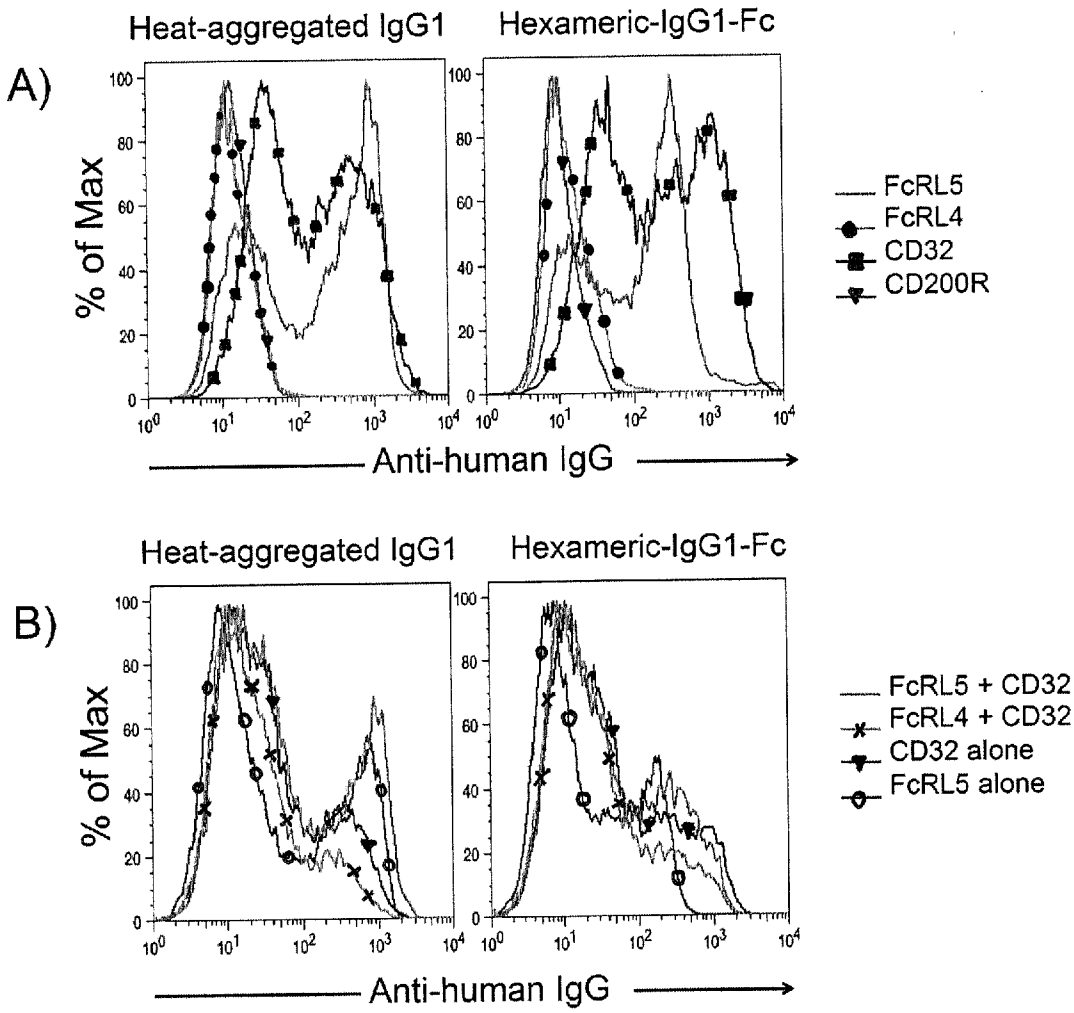
A)
Heat-aggregated IgG1
Hexameric-IgG1-Fc
% of Max
Anti-human IgG
FcRL5
FcRL4
CD32
CD200R
B)
Heat-aggregated IgG1
Hexameric-IgG1-Fc
% of Max
Anti-human IgG
FcRL5 + CD32
FcRL4 + CD32
CD32 alone
FcRL5 alone

Figure 3

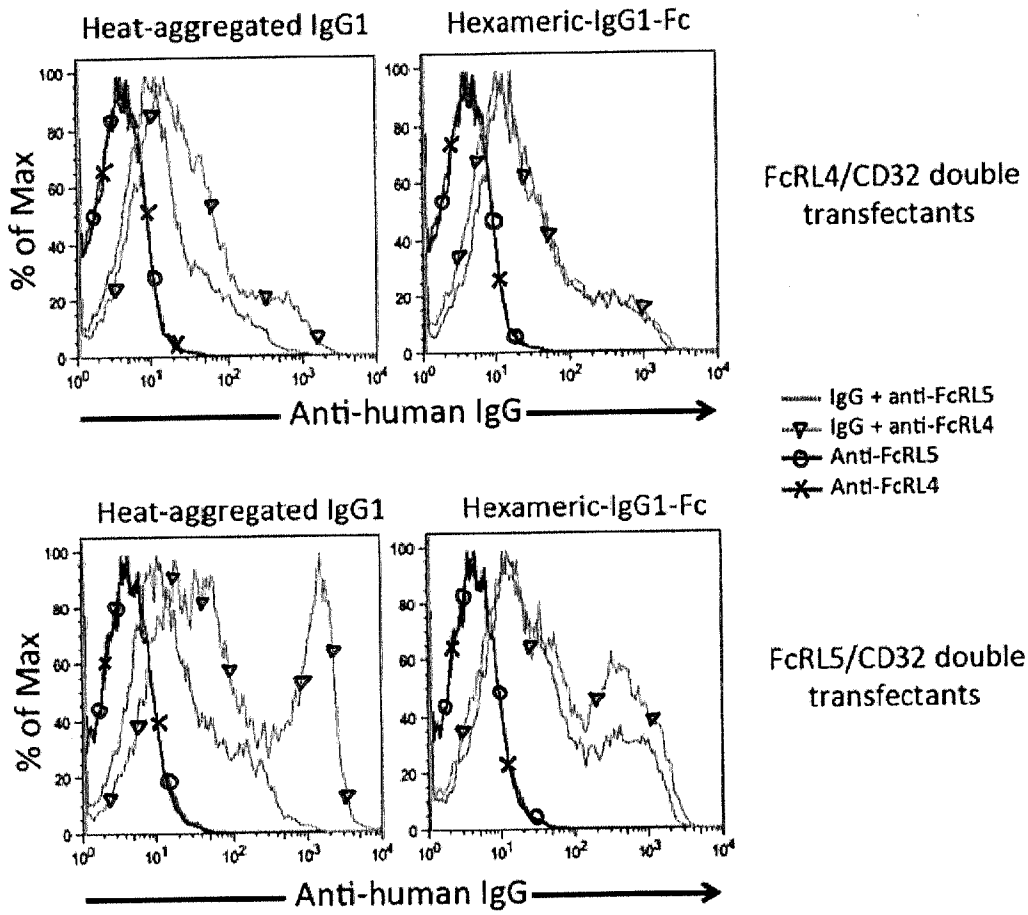
Heat-aggregated IgG1
Hexameric-IgG1-Fc
% of Max
Anti-human IgG
FcRL4/CD32 double transfectants
IgG + anti-FcRL5
IgG + anti-FcRL4
Anti-FcRL5
Anti-FcRL4
Heat-aggregated IgG1
Hexameric-IgG1-Fc
% of Max
Anti-human IgG
FcRL5/CD32 double transfectants

Figure 4

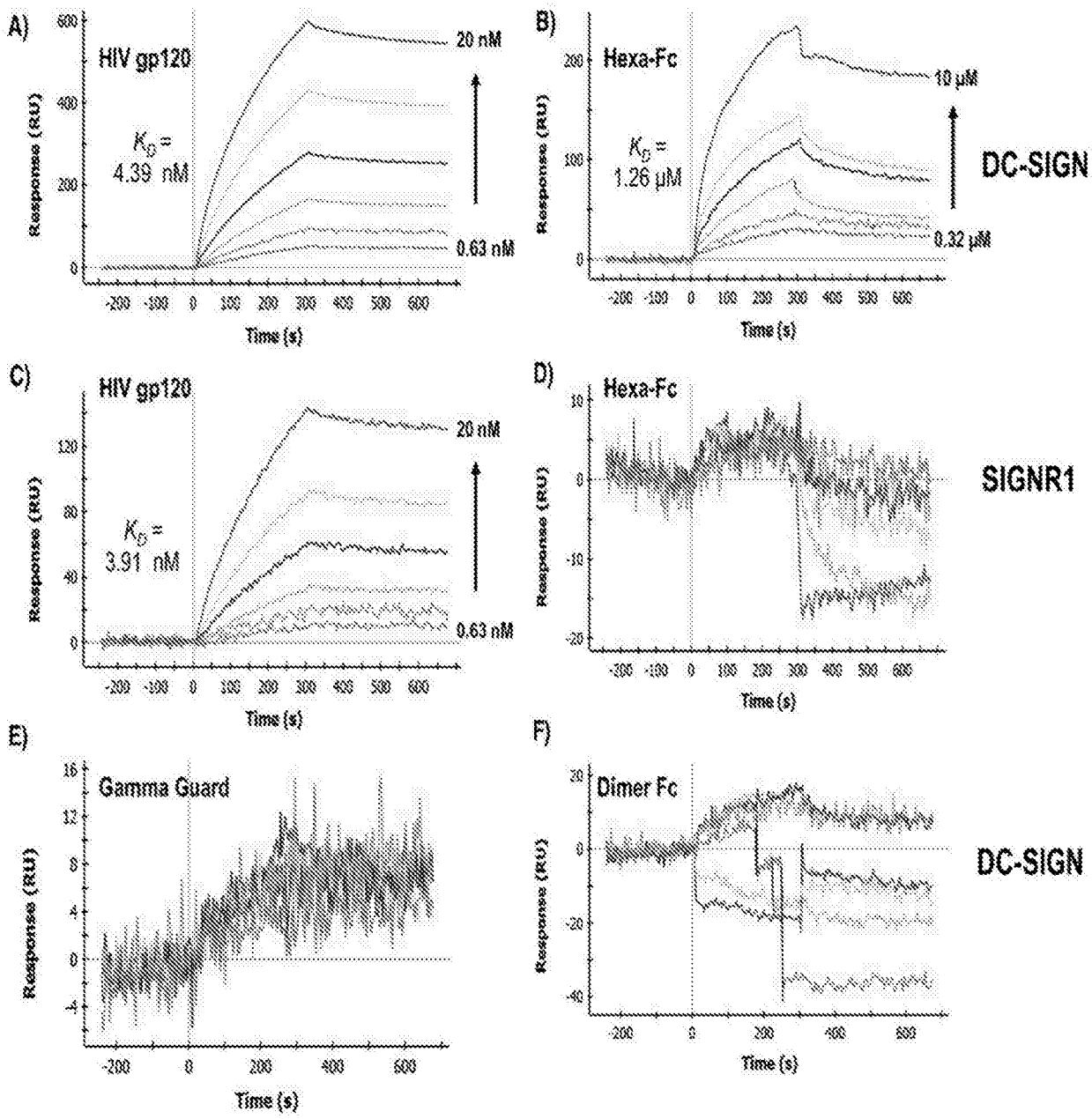
A)
HIV gp120
K_D = 4.39 nM
20 nM
0.63 nM
Response (RU)
Time (s)
B)
Hexa-Fc
K_D = 1.26 µM
10 µM
0.32 µM
DC-SIGN
Response (RU)
Time (s)
C)
HIV gp120
K_D = 3.91 nM
20 nM
0.63 nM
Response (RU)
Time (s)
D)
Hexa-Fc
SIGNR1
Response (RU)
Time (s)
E)
Gamma Guard
Response (RU)
Time (s)
F)
Dimer Fc
DC-SIGN
Response (RU)
Time (s)

Figure 5

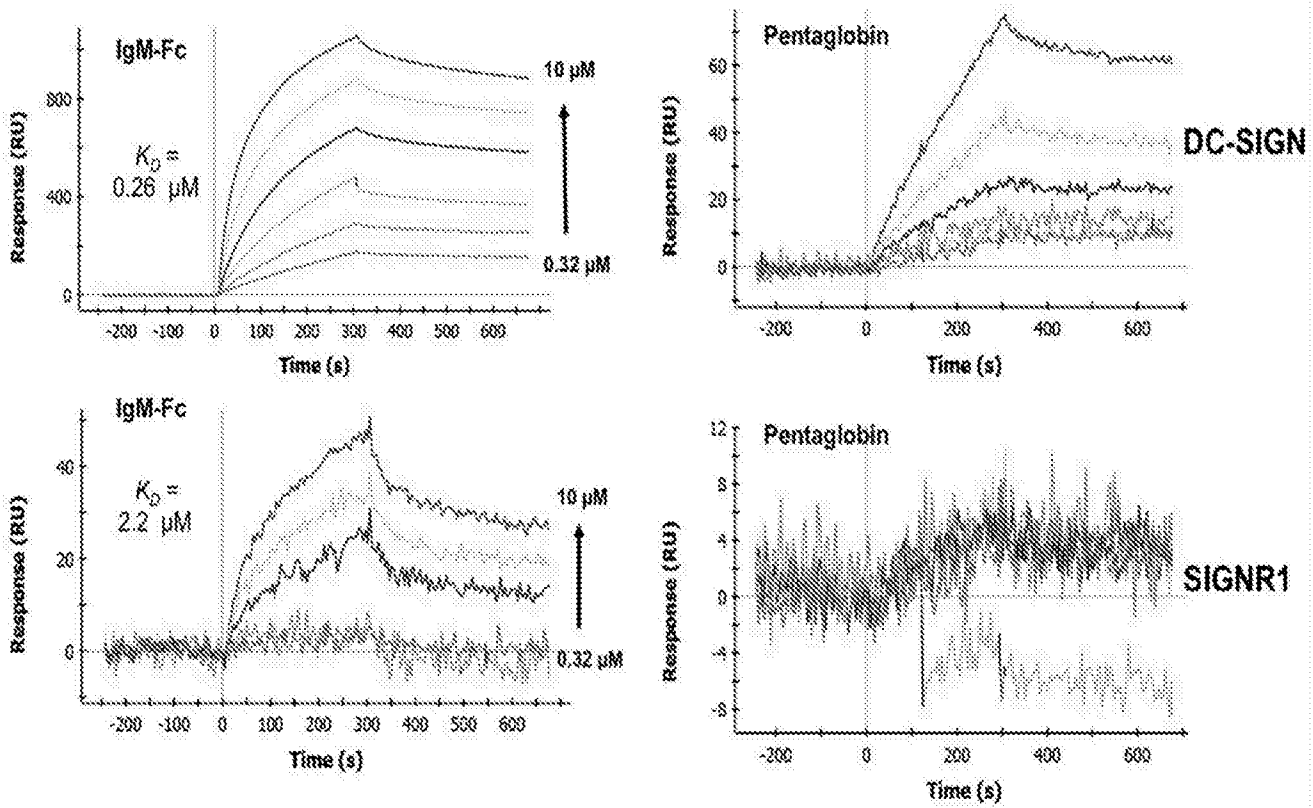
IgM-Fc
K_D = 0.26 µM
10 µM
0.32 µM
Response (RU)
Time (s)
Pentaglobin
DC-SIGN
IgM-Fc
K_D = 2.2 µM
10 µM
0.32 µM
Pentaglobin
SIGNR1

Figure 6

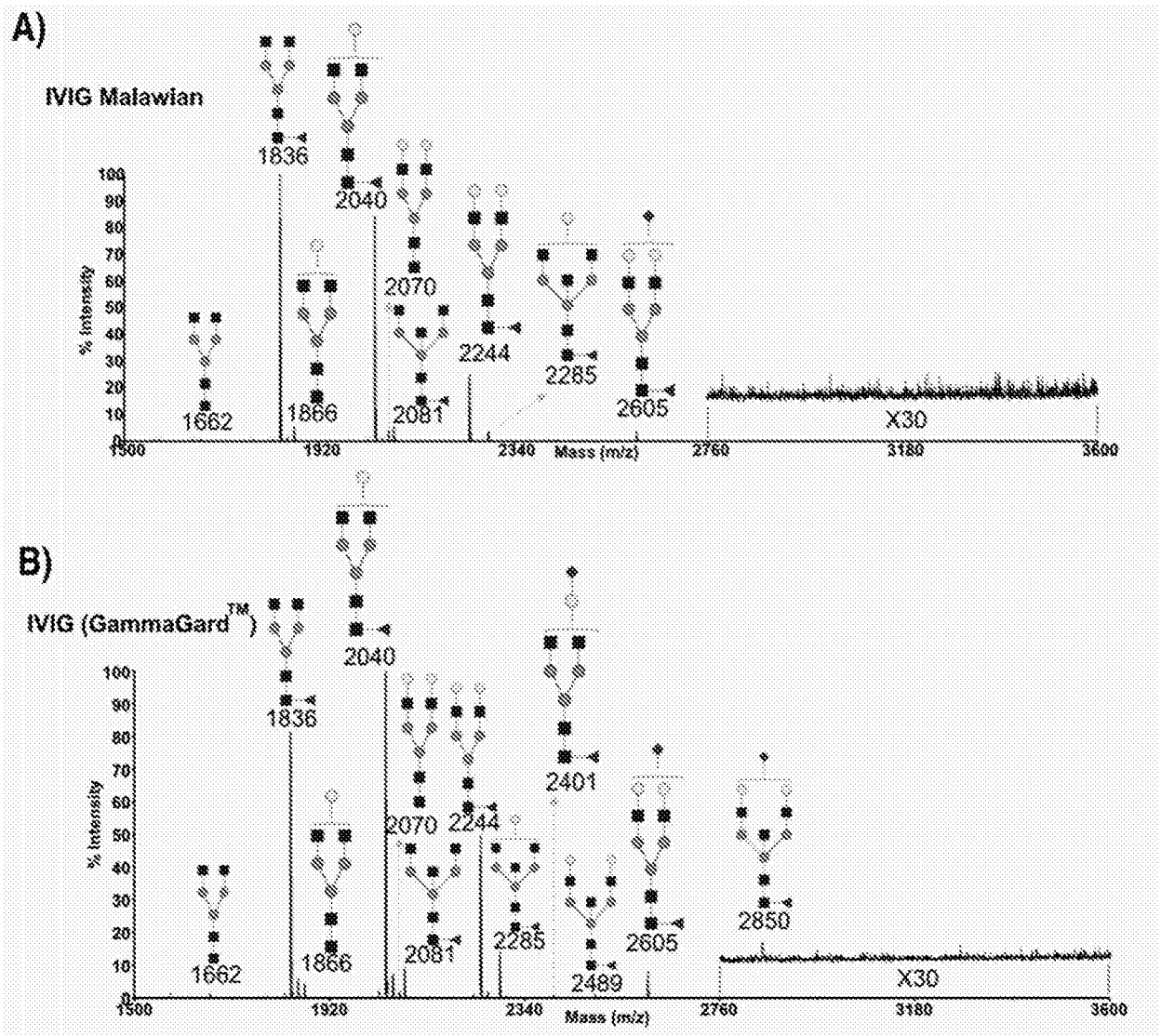
A)
IVIG Malawian
1836
2040
2070
2244
2285
2605
1662
1866
2081
X30
% intensity
Mass (m/z)
B)
IVIG (GammaGard™)
2040
1836
2401
2070
2244
2850
2285
2605
1662
1866
2081
2489
X30
% intensity
Mass (m/z)

Figure 7a

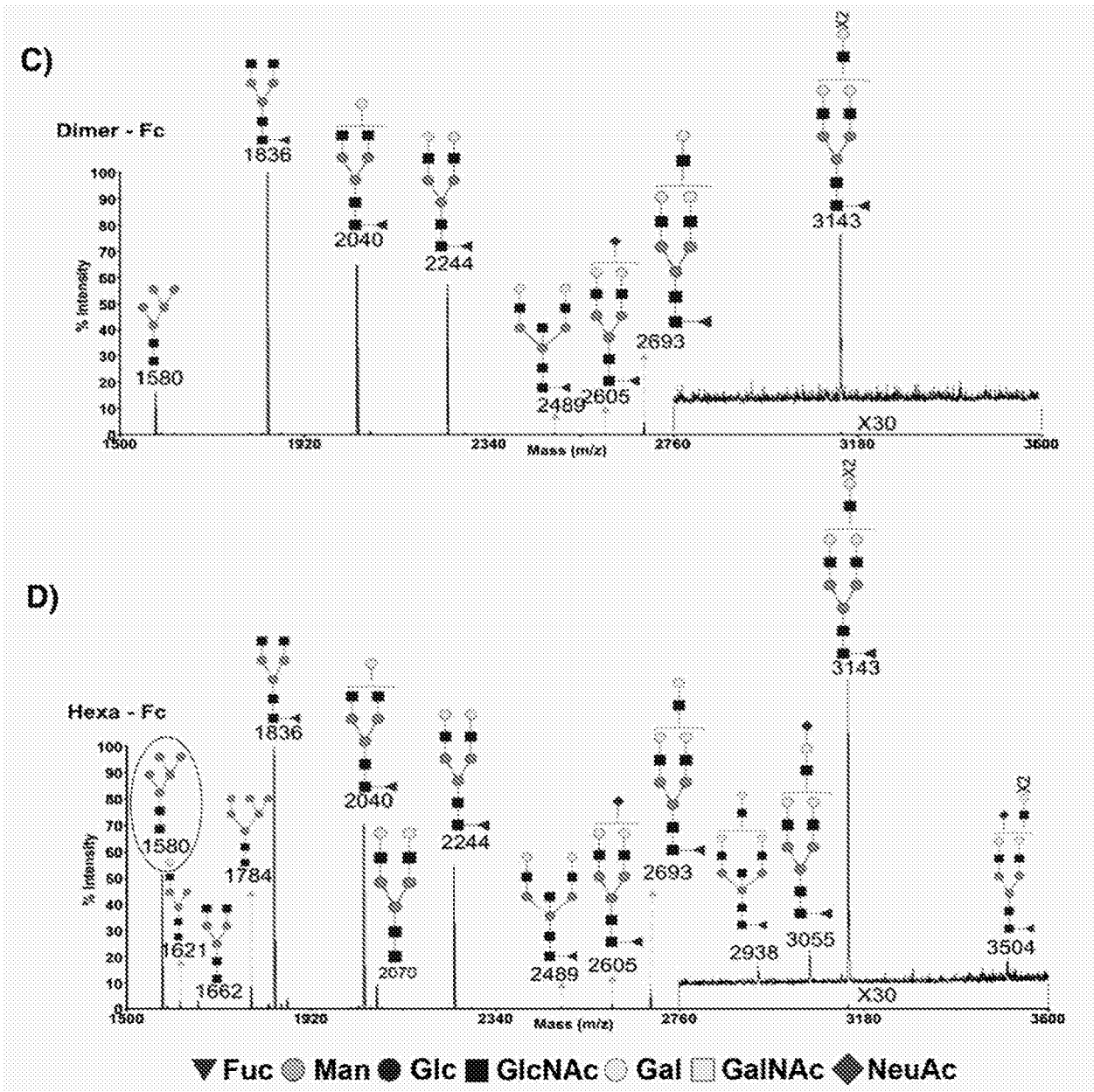
C)
Dimer - Fc
1836
2040
2244
3143
1580
2693
2489
2605
X30
% Intensity
Mass (m/z)
D)
Hexa - Fc
1836
2040
3143
1580
1784
2244
2693
1621
1662
2070
2489
2605
2938
3055
3504
X30
% Intensity
Mass (m/z)
Fuc Man Glc GlcNAc Gal GalNAc NeuAc

Figure 7a

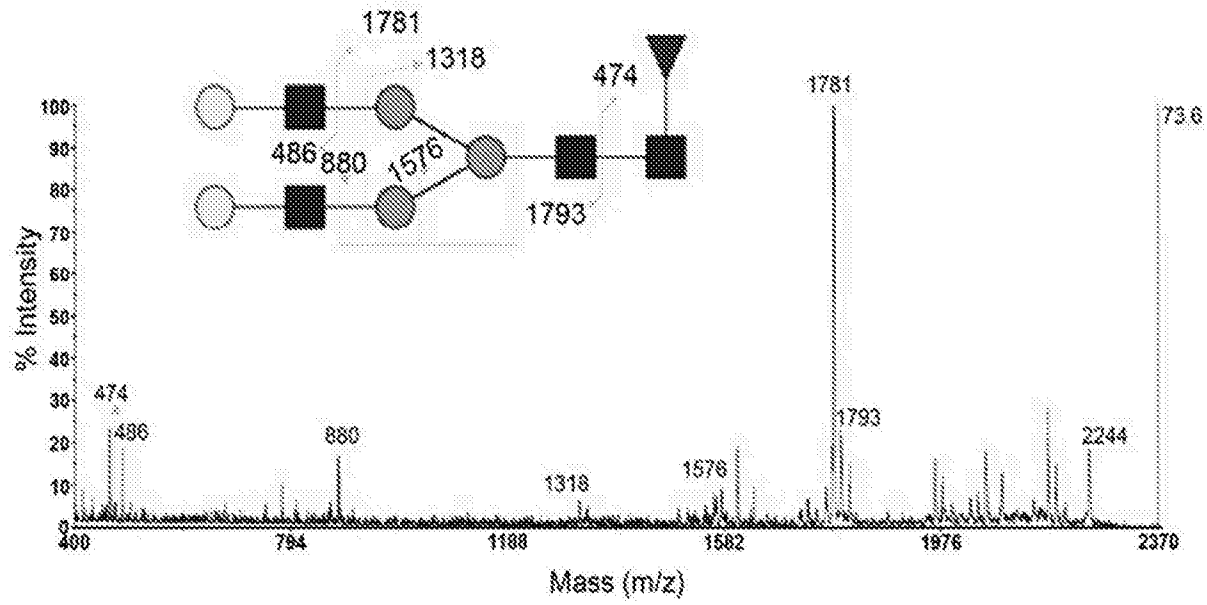
1781
1318
474
486
880
1576
1793
% Intensity
100
90
80
70
60
50
40
30
20
10
0
474
486
880
1318
1576
1781
1793
2244
736
400
794
1188
1582
1976
2370
Mass (m/z)

Figure 7B

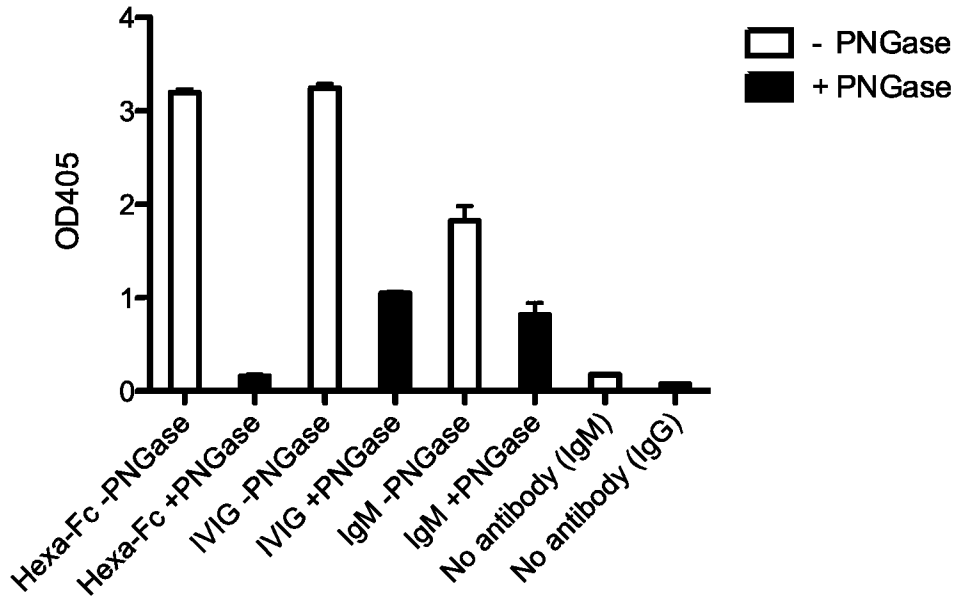
- PNGase
+ PNGase
OD405
4
3
2
1
0
Hexa-Fc -PNGase
Hexa-Fc +PNGase
IVIG -PNGase
IVIG +PNGase
IgM -PNGase
IgM +PNGase
No antibody (IgM)
No antibody (IgG)

Figure 8

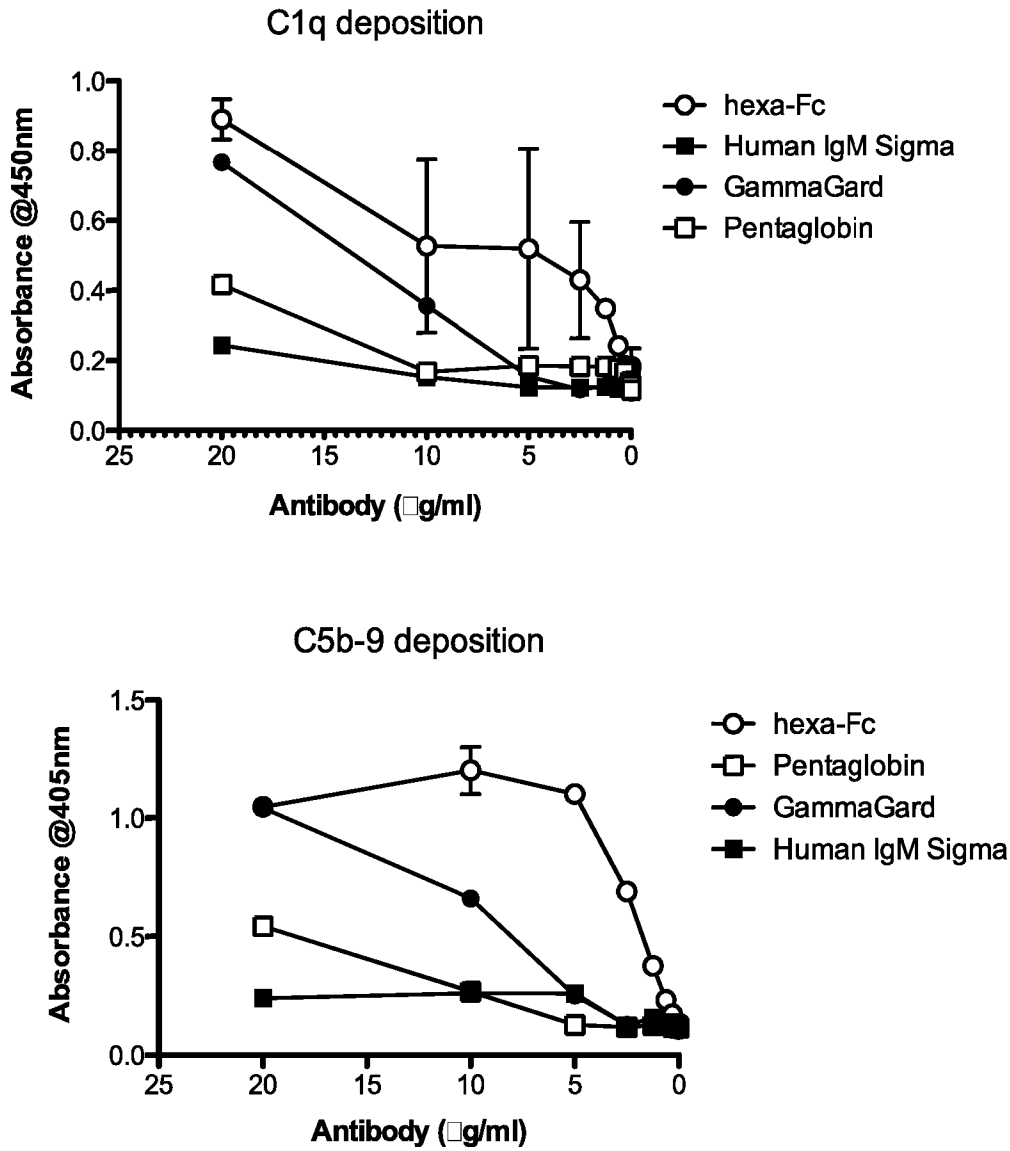
C1q deposition
Absorbance @450nm
1.0
0.8
0.6
0.4
0.2
0.0
25
20
15
10
5
0
Antibody (□g/ml)
hexa-Fc
Human IgM Sigma
GammaGard
Pentaglobin
C5b-9 deposition
Absorbance @405nm
1.5
1.0
0.5
0.0
25
20
15
10
5
0
Antibody (□g/ml)
hexa-Fc
Pentaglobin
GammaGard
Human IgM Sigma

Figure 9

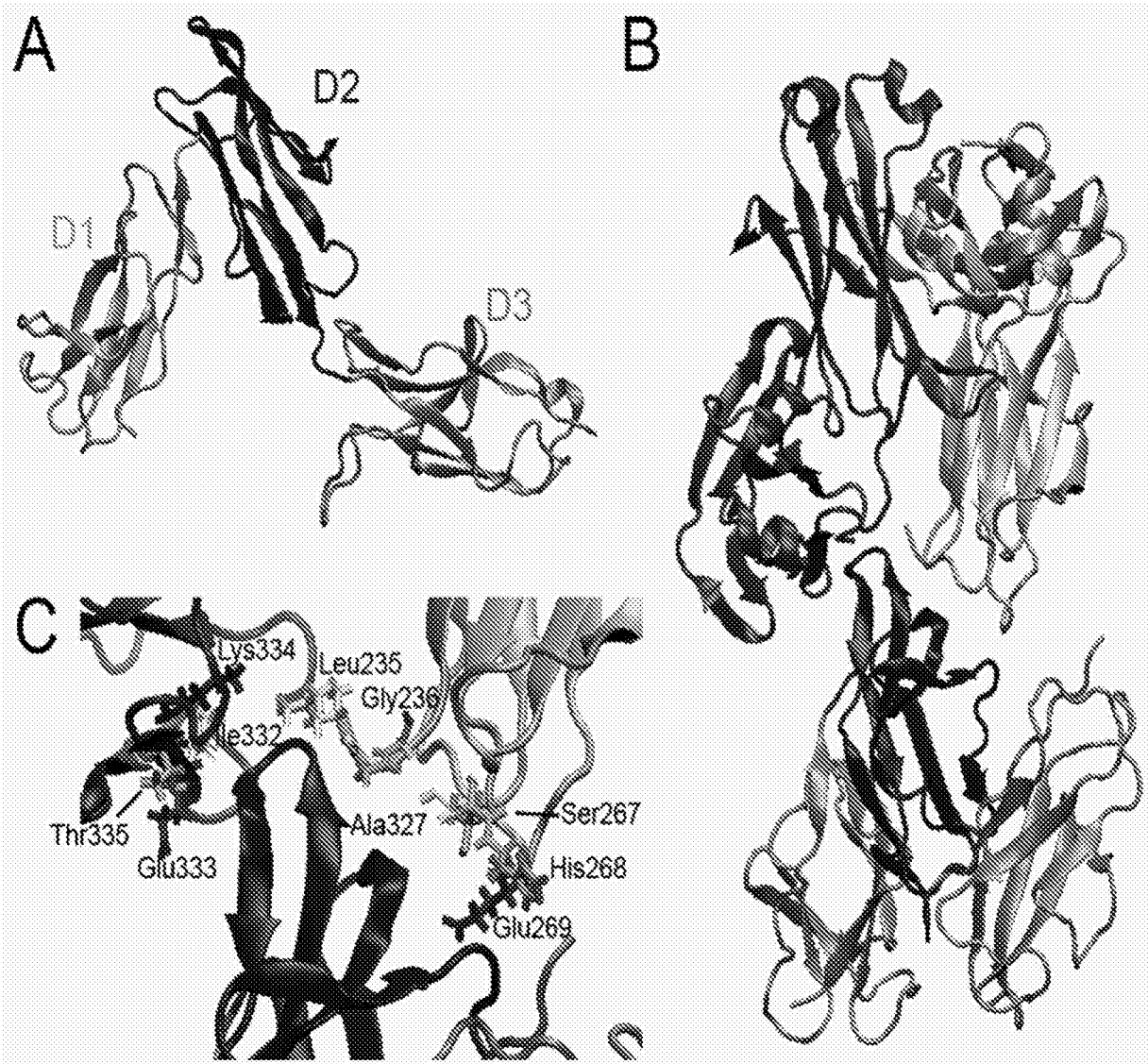
A
D2
D1
D3
B
C
Lys334
Leu235
Gly236
Ile332
Thr335
Glu333
Ala327
Ser267
His268
Glu269

Figure 10

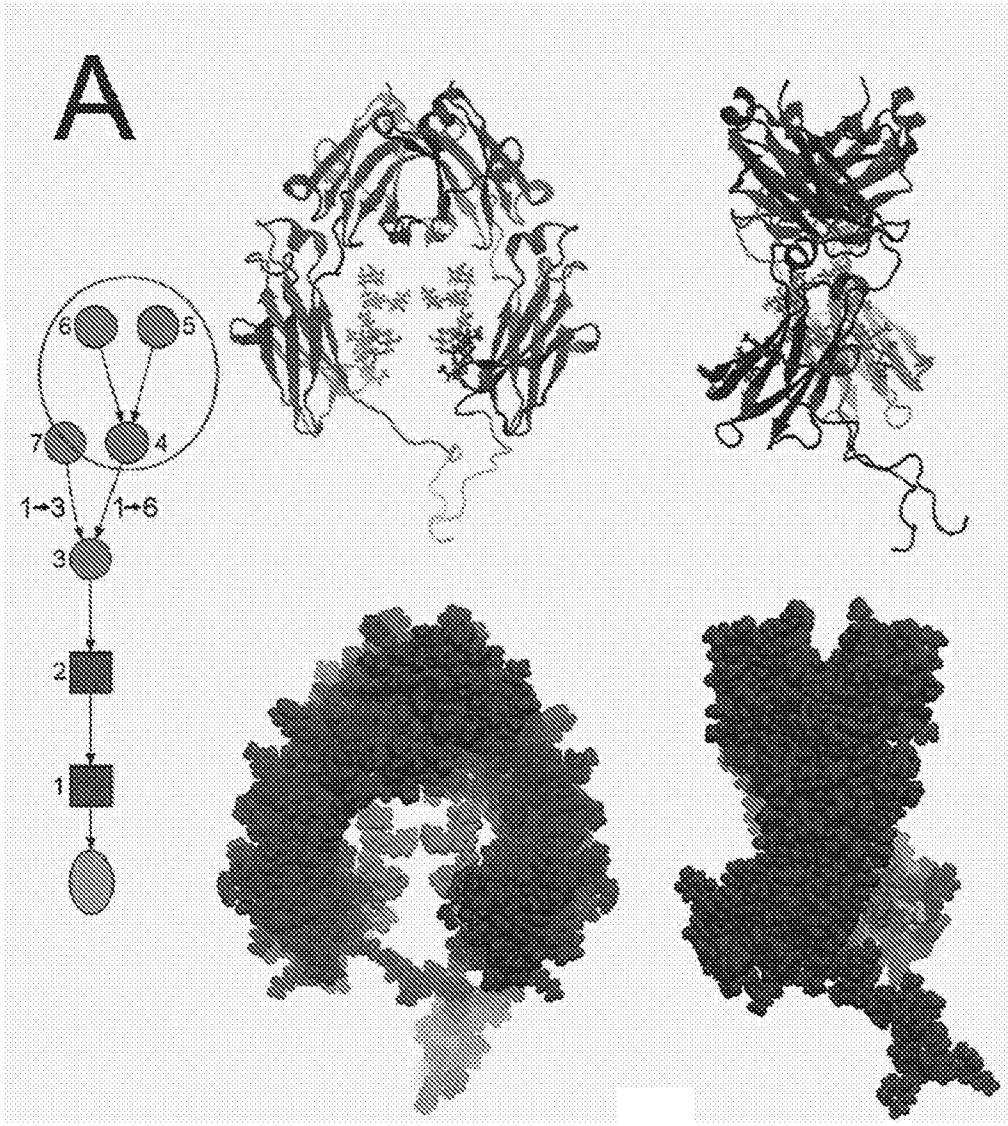

Figure 11

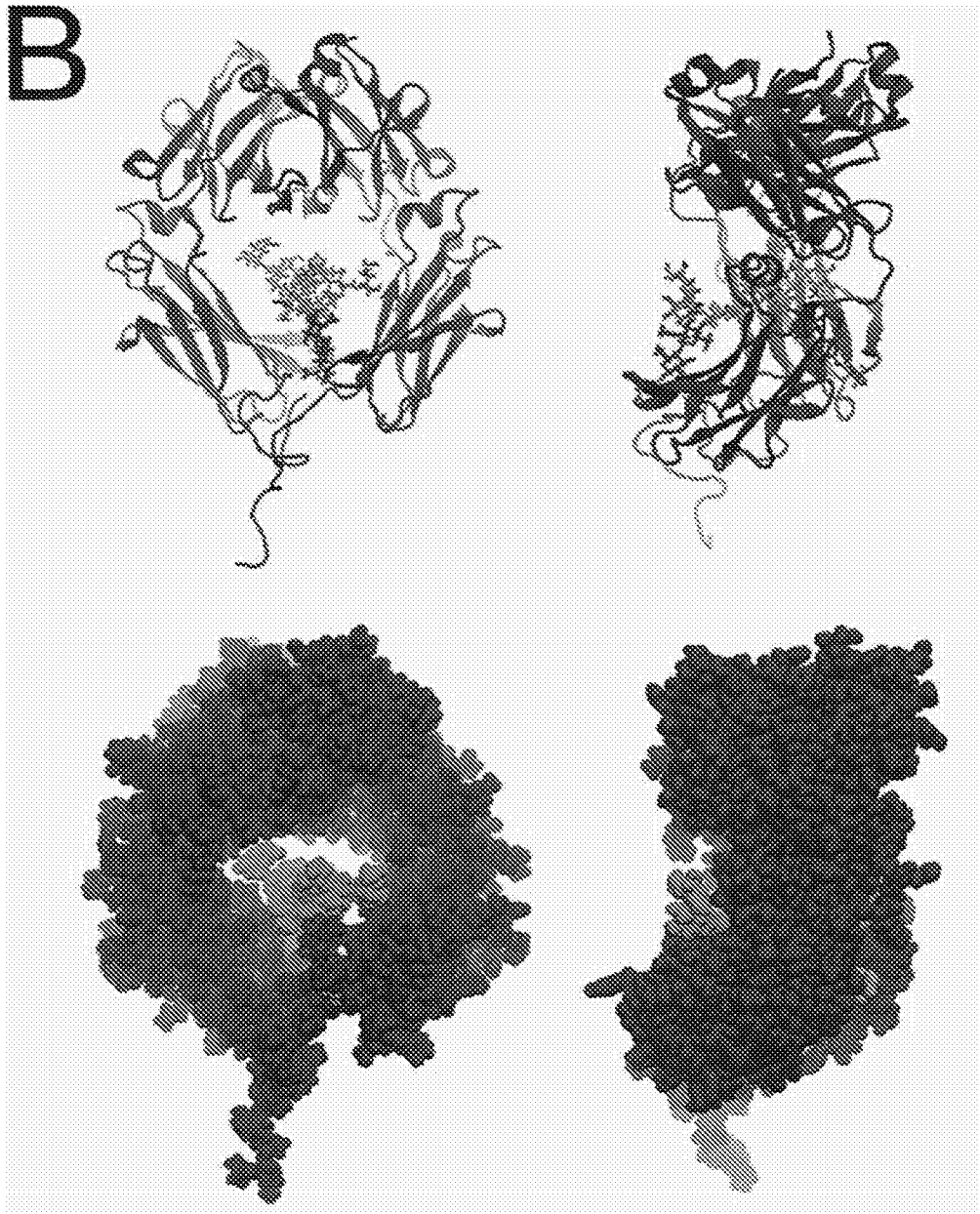

Figure 11

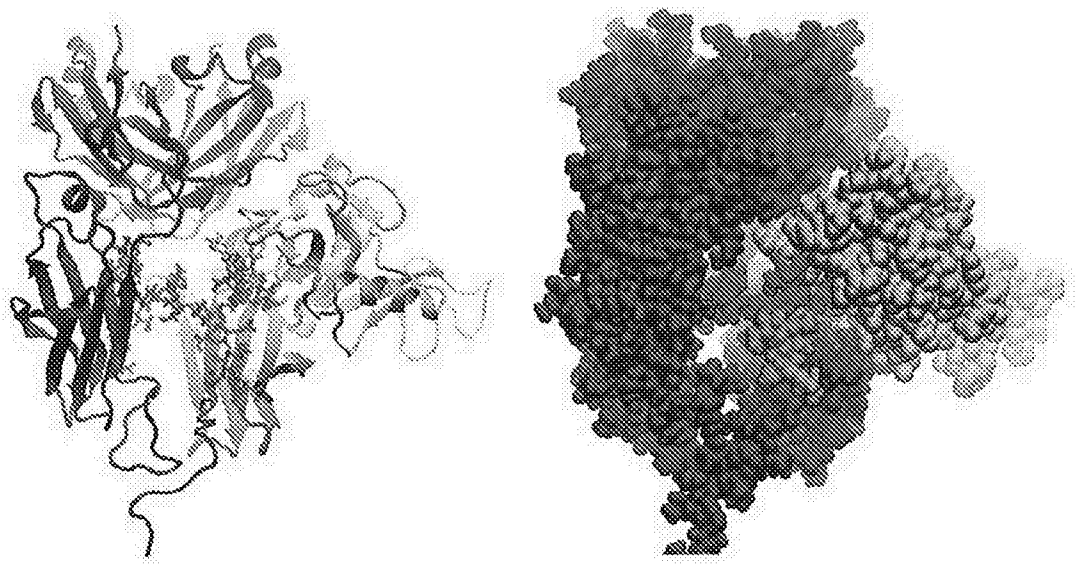

Figure 12

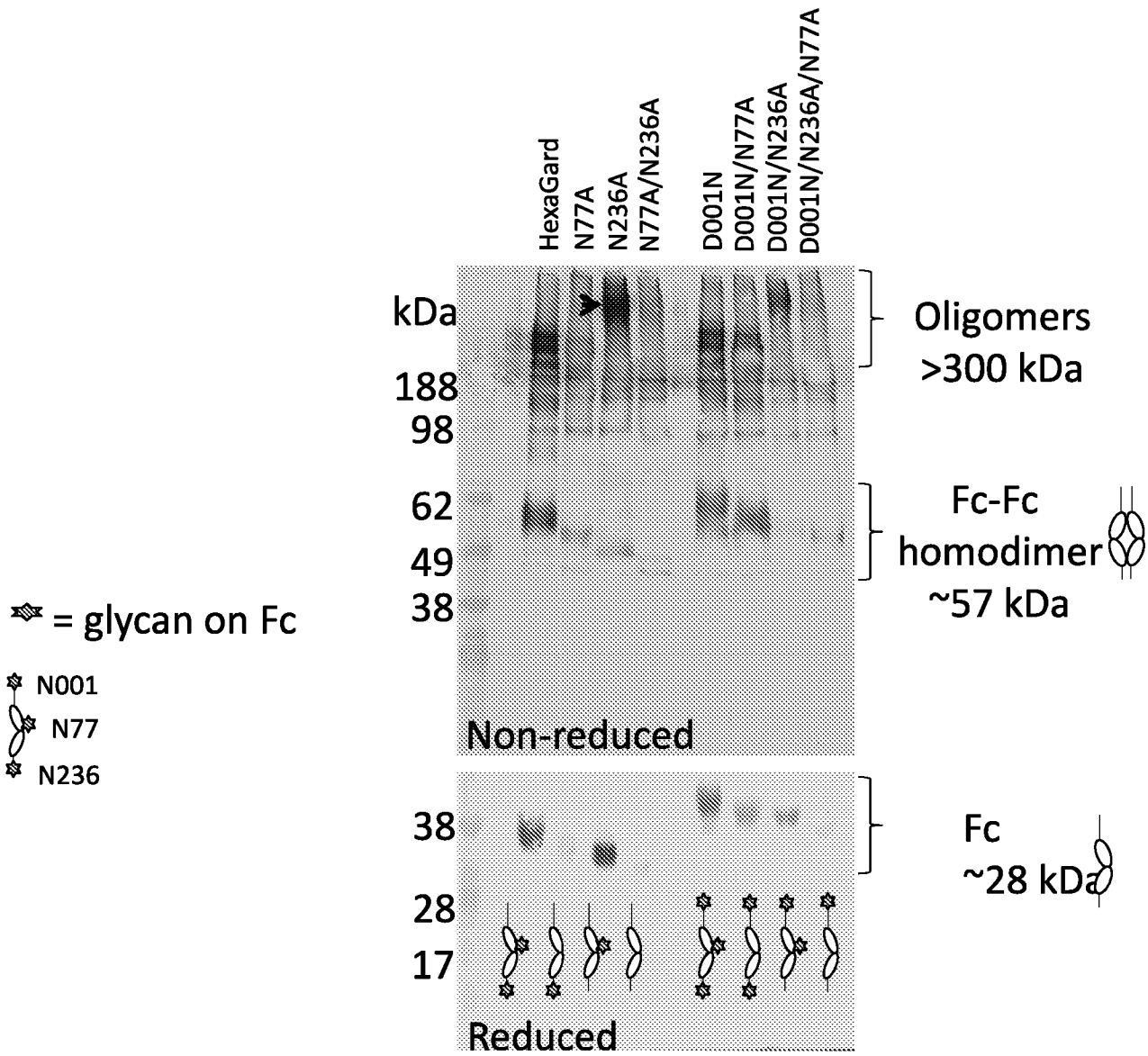
HexaGard
N77A
N236A
N77A/N236A
D001N
D001N/N77A
D001N/N236A
D001N/N236A/N77A
kDa
188
98
62
49
38
Oligomers
>300 kDa
Fc-Fc
homodimer
~57 kDa
Non-reduced
38
28
17
Fc
~28 kDa
Reduced
= glycan on Fc
N001
N77
N236

Figure 13

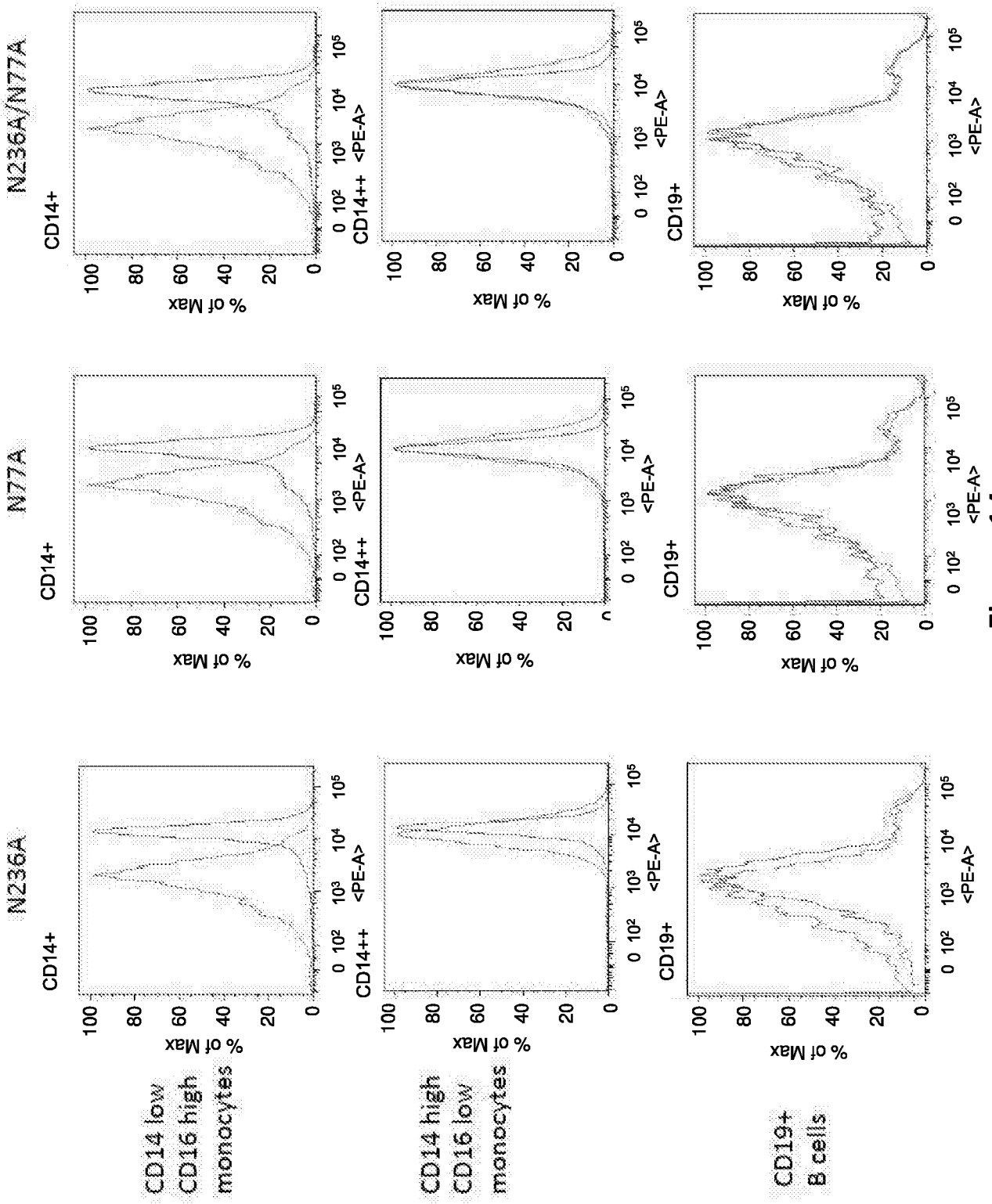
N236A
N77A
N236A/N77A
CD14+
CD14++
CD19+
% of Max
<PE-A>
CD14 low
CD16 high
monocytes
CD14 high
CD16 low
monocytes
CD19+
B cells

Figure 14

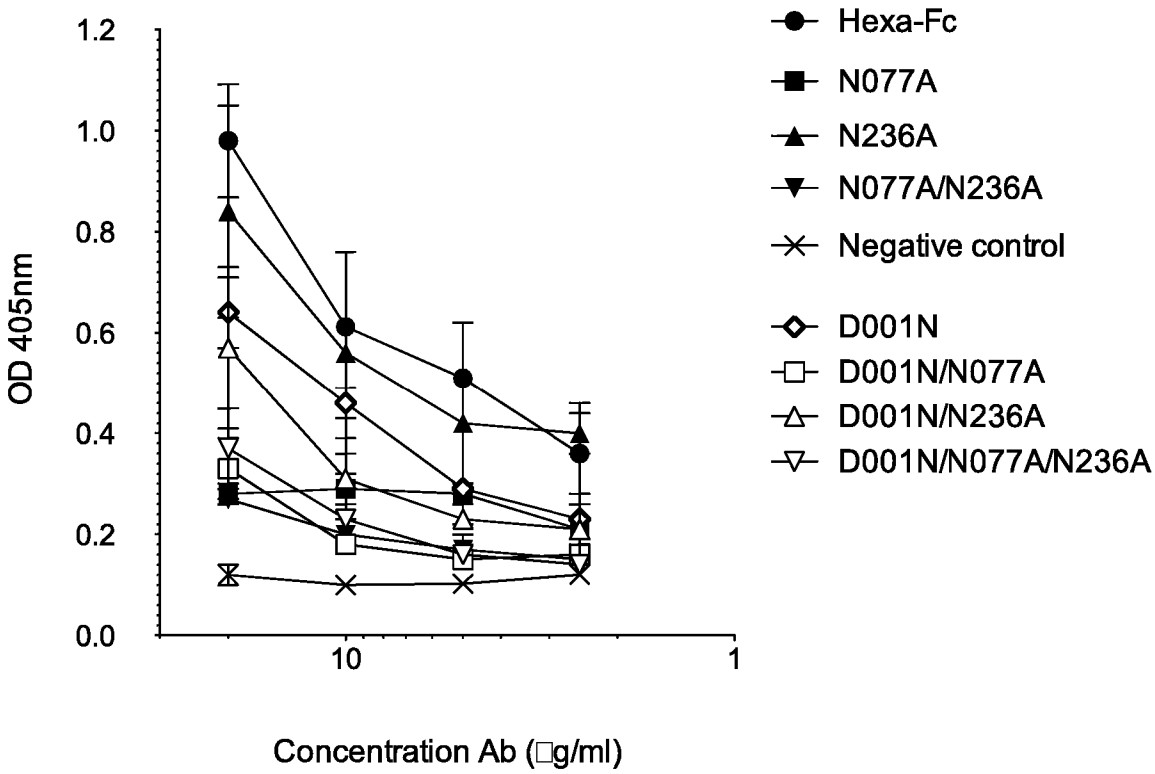
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 405nm
10
1
Concentration Ab (□g/ml)
Hexa-Fc
N077A
N236A
N077A/N236A
Negative control
D001N
D001N/N077A
D001N/N236A
D001N/N077A/N236A

Figure 15

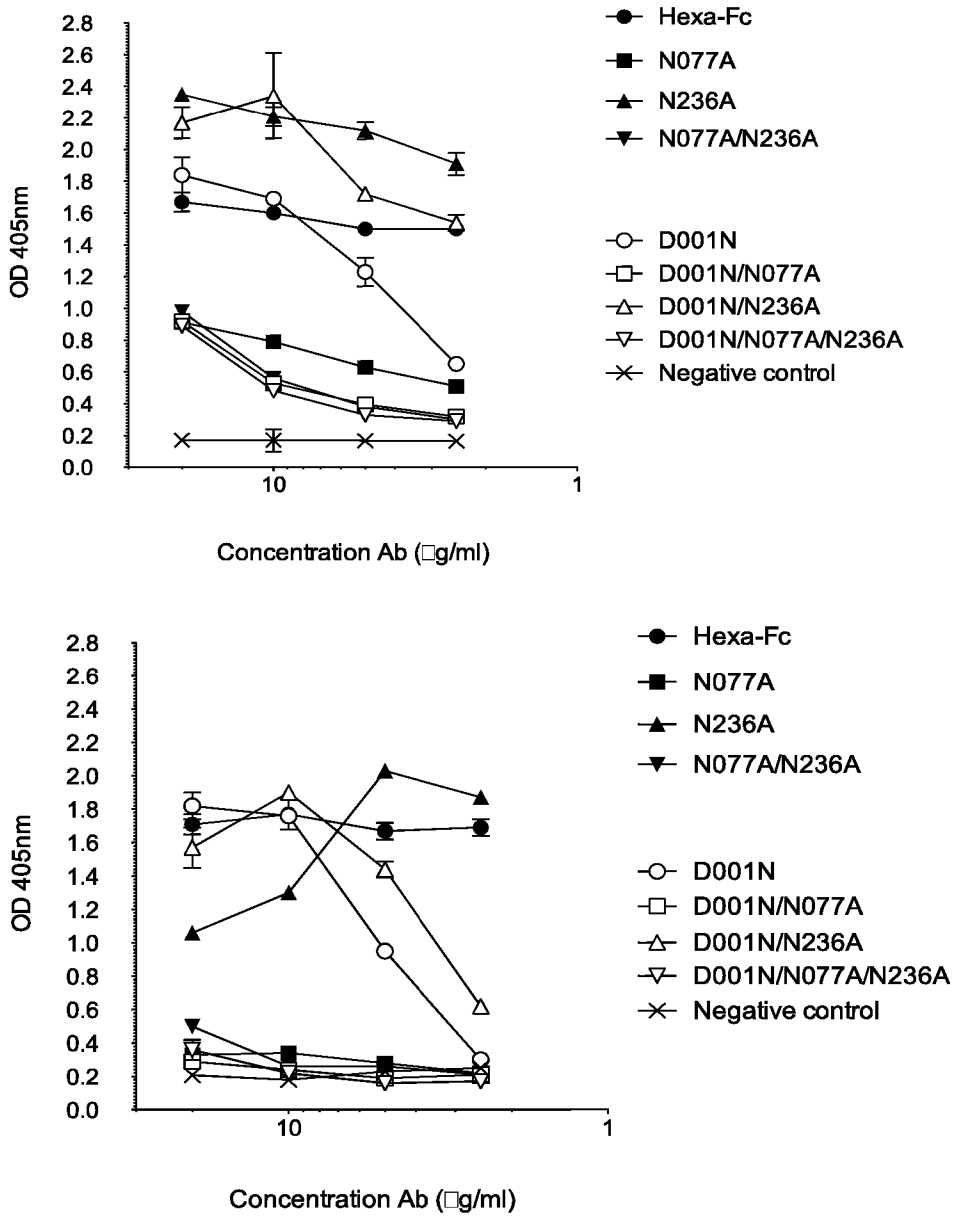
OD 405nm
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
1
Concentration Ab (□g/ml)
Hexa-Fc
N077A
N236A
N077A/N236A
D001N
D001N/N077A
D001N/N236A
D001N/N077A/N236A
Negative control

Figure 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/052098

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/46
ADD. A61K39/395

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	COLOMA M J ET AL: "The role of carbohydrate in the assembly and function of polymeric IgG.", MOLECULAR IMMUNOLOGY DEC 2000, vol. 37, no. 18, December 2000 (2000-12), pages 1081-1090, XP002745729, ISSN: 0161-5890 paragraphs [0001], [02.1] figure 1 page 1089, left-hand column, line 43 - line 47 page 1087, column 1089 ----- -/--	1-10, 13-31

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2015	20/10/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bumb, Peter

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2015/052098

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/052098

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SMITH R I ET AL: "Recombinant polymeric IgG: an approach to engineering more potent antibodies", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, GB, vol. 12, no. 7, 1 July 1994 (1994-07-01), pages 683-688, XP008176404, ISSN: 0733-222X figures 1-2	1-10, 13-31
Y	WO 2011/073692 A1 (UNIV NOTTINGHAM [GB]; PLEASS RICHARD JOHN [GB]) 23 June 2011 (2011-06-23) the whole document	1-10, 13-31

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2015/052098

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011073692 A1	23-06-2011	NONE	