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DESCRIPTION

FIELD

[0001] The present disclosure provides sialic acid binding molecules and uses (including medical uses) of the same.

BACKGROUND

[0002] Sialic-acid binding carbohydrate binding modules (CBMs) (including those derived from *Vibrio cholerae* and *Streptococcus pneumoniae* and multi-valent forms thereof) have been shown to be effective antimicrobial agents with some shown to protect mice against a lethal challenge of H7N9 influenza virus. The biologic, Sp2CBMTD, contains two copies of a CBM Family 40 domain from S. *pneumoniae* neuraminidase A (*Sp*CBM) genetically fused to the trimerization domain from *Pseudomonas aeruginosa* pseudaminidase (PaTD). The protein assembles via the trimerization domain to form an oligomer containing six copies of SpCBM.

[0003] However, with any protein-based therapeutic, there is a risk of adverse effects associated with the patient's immune responses to the biologic. In some cases, the biologic may induce the production of anti-drug antibodies (ADAs) which can result in the formation of immune complexes (ICs) between the ADA and the drug. The presence of such complexes may reduce the effectiveness or alter the half-life of the therapeutic, or could cause adverse effects such as hypersensitivity reactions.

[0004] There are a number of factors that influence the immunogenicity of a protein. One key determinant is the presence of intrinsic epitopes that can trigger T-cell dependent events that lead to antibody production. T-cells recognize short peptide fragments derived from the processing of the protein antigen that occurs within an antigen-presenting cell (APC). Some of these fragments will form complexes with human leukocyte antigen (HLA)/major histocompatibility complex (MHC) class II molecules, and, if bound stably enough, will be carried to the surface of the APC and presented to T-cells. The recognition of non-self peptide by the T-cell receptor triggers the subsequent immune response.

SUMMARY

[0005] Aspects of the present invention are set out in the appended claims. Any embodiments or examples which do not fall under the scope of the appended claims are provided by way of background reference.

[0006] The sialic acid binding molecules described herein may comprise one or more modified carbohydrate binding modules (CBMs).

[0007] As stated, with any protein-based therapeutic, there is the risk of adverse effects associated with the patient's immune responses to the biologic. The biologic may elicit the production of anti-drug antibodies (ADAs) which can result in the formation of immune complexes (ICs) between the ADA and the drug. The presence of such complexes may reduce the effectiveness, alter the half-life of the therapeutic or could cause adverse effects such as hypersensitivity reactions. The disclosed CBMs have been subject to modification so as to reduce the risk of these adverse effects.

[0008] Carbohydrate binding modules are classified into families and CBMs classed as members of the Family 40 CBMs (CBM40) may be useful in the manufacture of the modified molecules described herein. The Family 40 CBMs embrace molecules of approximately 200 residues and are often found at the N-terminus of GH33 sialidases. They may also be found inserted in the β-propeller of GH33 sialidases.

[0009] As such, the sialic acid binding molecules described herein may comprise one or more modified Family 40 carbohydrate binding modules (CBM40s).

[0010] Throughout this specification the term "comprising" is used to denote that embodiments "comprise" the noted features and as such, may also include other features. However, the term "comprising" may also encompass embodiments which "consist essentially of" the relevant features or "consist of" the relevant features.

[0011] In view of the above, the disclosure may embrace molecules, for example, larger molecules, which comprise a sialic acid binding component. As stated, that sialic acid binding component (i.e. the sialic acid binding molecule) may itself comprise (consist of or consist essentially of) a modified CBM, for example, a modified CBM40. By way of (non-limiting) example, the molecules of this disclosure may not only exhibit an ability to bind sialic acid, but may also have one or more other functions. For example, the molecules may have enzymatic activity. For example, a useful molecule may comprise a modified CBM (as described herein) and exhibit some sialidase activity.

[0012] In oneexample, the CBM may not be provided as part of, or comprised within, a molecule with enzymatic (for example sialidase) activity.

[0013] A useful sialic acid binding molecule may be a fusion protein comprising an enzymatic portion and a sialic acid binding portion - wherein the sialic acid binding portion comprises a modified CBM as described herein. In such cases, the enzymatic portion may be fused to the sialic acid binding portion. As stated, the enzymatic portion of any useful fusion protein may comprise (or have, or exhibit) sialidase activity.

[0014] The term "modified" embraces molecules which contain one or more mutations relative to a reference sequence.

[0015] A "reference sequence" may be any wild type CBM sequence. For example, a reference sequence may comprise, consist essentially of or consist of a wild type family 40 CBM sequence, e.g. the wild type CBM sequences from *Vibrio cholerae* NanH sialidase or *Streptococcus pneumoniae* NanA sialidase (it should be appreciated that similar or homologous CBMs (including CBM40s) present in other organisms are to be encompassed within the scope of the term "CBM" and/or as CBM reference sequences).

Accordingly, a modified CBM sequence may be derived from a specific or particular wild type CBM.

[0016] A modified CBM sequence may comprise a wild type CBM sequence which includes one or more mutations.

[0017] The one or more mutations may be functional - that is to say they may individually (and/or independently) or collectively (for example, synergistically) modulate (alter, improve or suppress/inhibit) one or more of the physiological, biological immunological and/or pharmacological properties characteristic of a wild type CBM (for example the wild type CBM from which the modified CBM is derived). In particular, the one or more mutations may:

1. (i) alter the immunogenicity (or antigenicity) of the CBM; and/or
2. (ii) alter (for example improve) the efficacy (of the CBM or of any multimeric molecule comprising a modified CBM)' and/or
3. (iii) they may modulate (for example improve) the thermostability of the CBM; and/or
4. (iv) they may modulate (for example improve) the solubility of the CBM; and/or
5. (v) they may modulate (for example improve) the *in vivo* half-life of the molecule.

[0018] A "mutation" may include any alteration to the wild-type CBM molecule. For example, the term "mutation" may embrace, for example:

1. (i) one or more amino acid substitution(s) (where one or more of the wild type amino acid(s) is/are swapped or changed for another (different) amino acid - the term "substitutions" would include conservative amino acid substitutions); and/or
2. (ii) one or more amino acid deletion(s) (where one or more of the wild type amino acid residue(s) are removed); and/or
3. (iii) one or more amino acid addition(s)/insertion(s) (where additional amino acid residue(s) are added to a wild type (or reference) primary sequence); and/or
4. (iv) one or more amino acid/sequence inversions (usually where two or more consecutive amino acids in a primary sequence are reversed; and/or
5. (v) one or more amino acid/sequence duplications (where an amino acid or a part of the primary amino acid sequence (for example a stretch of 5-10 amino acids) is repeated)

[0019] As stated, a modified CBM according to this disclosure may comprise one or more of the mutations described herein.

[0020] An exemplary wild type CBM (in other words a reference sequence from which a useful modified CBM may be derived) is *Streptococcus pneumoniae* NanA sialidase - the amino acid sequence for which has been deposited under accession number P62575 and is reproduced below as SEQ ID NO: 1 (1035 amino acids).

SEQ ID NO: 1

MSYFRNRDID IERNSMNRSV QERKCRYSIR KLSVGAVSMI VGAVVFGTSP VLAQEGASEQ

PLANETQLSG ESSTLTDTEK SQPSSETELS GNKQEQERKD KQEEKIPRDY YARDLENVET

VIEKEDVETN ASNGQRVDLS SELDKLKKLE NATVHMEFKP DAKAPAFYNL FSVSSATKKD

EYFTMAVYNN TATLEGRGSD GKQFYNNYND APLKVKPGQW NSVTFTVEKP TAELPKGRVR

LYVNGVLSRT SLRSGNFIKD MPDVTHVQIG ATKRANNTVW GSNLQIRNLT VYNRALTPEE

VQKRSQLFKR SDLEKKLPEG AALTEKTDIF ESGRNGKPNK DGIKSYRIPA LLKTDKGTLI

AGADERRLHS SDWGDIGMVI RRSEDNGKTW GDRVTITNLR DNPKASDPSI GSPVNIDMVL

VQDPETKRIF SIYDMFPEGK GIFGMSSQKE EAYKKIDGKT YQILYREGEK GAYTIRENGT

VYTPDGKATD YRVVVDPVKP AYSDKGDLYK GNQLLGNIYF TTNKTSPFRI AKDSYLWMSY

SDDDGKTWSA PQDITPMVKA DWMKFLGVGP GTGIVLRNGP HKGRILIPVY TTNNVSHLNG

SQSSRIIYSD DHGKTWHAGE AVNDNRQVDG QKIHSSTMNN RRAQNTESTV VQLNNGDVKL

FMRGLTGDLQ VATSKDGGVT WEKDIKRYPQ VKDVYVQMSA IHTMHEGKEY IILSNAGGPK

RENGMVHLAR VEENGELTWL KHNPIQKGEF AYNSLQELGN GEYGILYEHT EKGQNAYTLS

FRKFNWDFLS KDLISPTEAK VKRTREMGKG VIGLEFDSEV LVNKAPTLQL ANGKTARFMT

QYDTKTLLFT VDSEDMGQKV TGLAEGAIES MHNLPVSVAG TKLSNGMNGS EAAVHEVPEY

TGPLGTSGEE PAPTVEKPEY TGPLGTSGEE PAPTVEKPEY TGPLGTAGEE AAPTVEKPEF

TGGVNGTEPA VHEIAEYKGS DSLVTLTTKE DYTYKAPLAQ QALPETGNKE SDLLASLGLT

AFFLGLFTLG KKREQ

[0021] The CBM region of SEQ ID NO: 1 is from amino acid residue 121 to 305 - this sequence is designated SEQ ID NO: 2 (that sequence being: VIEKEDVETN ASNGQRVDLS SELDKLKKLE NATVHMEFKP DAKAPAFYNL FSVSSATKKD EYFTMAVYNN TATLEGRGSD GKQFYNNYND APLKVKPGQW NSVTFTVEKP TAELPKGRVR LYVNGVLSRT SLRSGNFIKD MPDVTHVQIG ATKRANNTVW GSNLQIRNLT VYNRALTPEE VQKRS) .

[0022] Thus this disclosure provides sialic acid binding molecules which comprise modified forms of SEQ ID NO: 2 (and/or SEQ ID NO: 1). A modified form of SEQ ID NO: 2 may comprise one or more mutated residues - the mutations being, for example, amino acid substitutions, additions/insertions, duplications, deletions and/or inversions made relative to the sequence of SEQ ID NO: 2.

[0023] An exemplary *Vibrio cholerae* NanH sialidase amino acid sequence is deposited under accession umber A5F7A4 and is reproduced below as SEQ ID NO: 3 (781 amino acids).

SEQ ID NO: 3

MRFKNVKKTA LMLAMFGMAT SSNAALFDYN ATGDTEFDSP AKQGWMQDNT NNGSGVLTNA

DGMPAWLVQG IGGRAQWTYS LSTNQHAQAS SFGWRMTTEM KVLSGGMITN YYANGTQRVL

PIISLDSSGN LVVEFEGQTG RTVLATGTAA TEYHKFELVF LPGSNPSASF YFDGKLIRDN

IQPTASKQNM IVWGNGSSNT DGVAAYRDIK FEIQGDVIFR GPDRIPSIVA SSVTPGVVTA

FAEKRVGGGD PGALSNTNDI ITRTSRDGGI TWDTELNLTE QINVSDEFDF SDPRPIYDPS

SNTVLVSYAR WPTDAAQNGD RIKPWMPNGI FYSVYDVASG NWQAPIDVTD QVKERSFQIA

GWGGSELYRR NTSLNSQQDW QSNAKIRIVD GAANQIQVAD GSRKYVVTLS IDESGGLVAN

LNGVSAPIIL QSEHAKVHSF HDYELQYSAL NHTTTLFVDG QQITTWAGEV SQENNIQFGN

ADAQIDGRLH VQKIVLTQQG HNLVEFDAFY LAQQTPEVEK DLEKLGWTKI KTGNTMSLYG

NASVNPGPGH GITLTRQQNI SGSQNGRLIY PAIVLDRFFL NVMSIYSDDG GSNWQTGSTL

PIPFRWKSSS ILETLEPSEA DMVELQNGDL LLTARLDFNQ IVNGVNYSPR QQFLSKDGGI

TWSLLEANNA NVFSNISTGT VDASITRFEQ SDGSHFLLFT NPQGNPAGTN GRQNLGLWFS

FDEGVTWKGP IQLVNGASAY SDIYQLDSEN AIVIVETDNS NMRILRMPIT LLKQKLTLSQ

N

[0024] The CBM region of SEQ ID NO: 3 is from amino acid residue 25 to 216 - this sequence may be SEQ ID NO: 4 (that sequence being: ALFDYNATGD TEFDSPAKQG WMQDNTNNGS GVLTNADGMP AWLVQGIGGR AQWTYSLSTN QHAQASSFGW RMTTEMKVLS GGMITNYYAN GTQRVLPIIS LDSSGNLVVE FEGQTGRTVL ATGTAATEYH KFELVFLPGS NPSASFYFDG KLIRDNIQPT ASKQNMIVWG NGSSNTDGVA AY)

[0025] Thus this disclosure provides sialic acid binding molecules which comprise modified forms of SEQ ID NO: 4 (and/or SEQ ID NO: 3). A modified form of SEQ ID NO: 4 may comprise one or more mutated residues - the mutations being, for example, amino acid substitutions, additions/insertions, duplications, deletions and/or inversions made relative to the sequence of SEQ ID NO: 4.

[0026] As stated, the sialic acid binding molecules described herein may comprise one or more (for example two or more) modified CBMs. Where the sialic acid binding molecules comprise two or more modified CBMs, the molecule may be referred to as a multivalent CBM. A sialic acid binding molecule which is (or which comprises) a multivalent CBM, may be prepared as a construct comprising the multiple (two or more) modified CBMs linked by amino acid/peptide linkers. Each modified CBM may be linked to another by, for example, peptides comprising 5, 10 or 15 amino acids. By way of example any one or more of the following peptides may be used to link two or

more CBMs to produce a sialic acid binding molecule which is a multivalent CBM:

(i) 5 amino acid linkers:	ALXGS
	LQALG
	GGXSG
	GGALG
	GGSLG
(ii) 10 amino acid linkers:	ALXGSGGGSG
	LQALGGGGSL
(iii) 15 amino acid linkers:	ALXGSGGGSGGGGSG

[0027] Where "X" is any amino acid.

[0028] The sialic acid binding molecules for use may further comprise one or more oligomerisation domain(s). Like the CBM component of the disclosed sialic acid binding molecules, the oligomerisation domains may be derived from wild type oligomerisation domains and may therefore comprise one or more mutations relative to a corresponding wild type oligomerisation domain sequence. Useful oligomerisation domains exhibit an ability to self-associate to form multimeric structures, for example, trimers. A modified oligomerisation domain for use may comprise any molecule with that same oligomerisation property. For the avoidance of doubt, the term "oligomerisation domain" as used herein embraces not only wild type oligomerisation domains, but also those that are modified (the "modified oligomerisation domains" disclosed herein).

[0029] For example, a sialic acid binding molecule according to this disclosure may comprise one or more (for example, two) CBMs (for example, one, two or more modified CBMs as described herein) bound, coupled or fused to an oligomerisation domain. The resulting sialic acid binding molecule is a (multimeric) CBM::oligomerisation domain "fusion".

[0030] Suitable oligomerisation domains may include those obtainable from the *Pseudomonas aeruginosa* pseudaminidase. An exemplary *Pseudomonas aeruginosa* pseudaminidase amino acid sequence has been deposited under accession number Q9L6G4 (derived from strain PAO579) and is reproduced below as SEQ ID NO: 13 (438 amino acids). It should be appreciated that similar, homologous or other useful oligomerisation domains may be encompassed within the scope of the general term "oligomerisation domain".

SEQ ID NO: 13

MNTYFDIPHR LVGKALYESY YDHFGQMDIL SDGSLYLIYR RATEHVGGSD GRVVFSKLEG

GIWSAPTIVA QAGGQDFRDV AGGTMPSGRI VAASTVYETG EVKVYVSDDS GVTWVHKFTL

ARGGADYNFA HGKSFQVGAR YVIPLYAATG VNYELKWLES SDGGETWGEG STIYSGNTPY

NETSYLPVGD GVILAVARVG SGAGGALRQF ISLDDGGTWT DQGNVTAQNG DSTDILVAPS

LSYIYSEGGT PHVVLLYTNR TTHFCYYRTI LLAKAVAGSS GWTERVPVYS APAASGYTSQ

VVLGGRRILG NLFRETSSTT SGAYQFEVYL GGVPDFESDW FSVSSNSLYT LSHGLQRSPR

RVVVEFARSS SPSTWNIVMP SYFNDGGHKG SGAQVEVGSL NIRLGTGAAV WGTGYFGGID

NSATTRFATG YYRVRAWI

[0031] The oligomerisation domain of SEQ ID NO: 13 is from amino acid residue 333 to 438 - this sequence may be SEQ ID NO: 14 (that sequence being: VPDFESDWFS VSSNSLYTLS HGLQRSPRRV VVEFARSSSP STWNIVMPSY FNDGGHKGSG AQVEVGSLNI RLGTGAAVWG TGYFGGIDNS ATTRFATGYY RVRAWI).

[0032] As stated, the disclosure may exploit modified oligomerisation domains. A modified oligomerisation domain may comprise an oligomerisation domain which contains one or more mutations relative to a reference oligomerisation sequence.

[0033] A "reference oligomerisation sequence" may be any wild type oligomerisation domain sequence, including for example SEQ ID NOS: 13 and 14 above.

[0034] Accordingly, a modified oligomerisation domain may be derived from a specific or particular wild type oligomerisation domain.

[0035] A modified oligomerisation domain sequence may comprise a wild type oligomerisation domain sequence which includes one or more mutations.

[0036] The one or more mutations may be functional - that is to say they may individually (and/or independently) or collectively (for example synergistically) modulate (improve or suppress/inhibit) one or more of the physiological, immunological, biological and/or pharmacological properties characteristic of a wild type oligomerisation domain (for example the wild type oligomerisation domain from which the modified oligomerisation domain is derived). As stated above, the mutation(s) may

1. (i) alter the immunogenicity (or antigenicity) of the oligomerisation domain; and/or
2. (ii) improve efficacy (of, for example, multimeric molecules comprising one or more modified CBMs); and/or
3. (iii) they may modulate (for example improve) the thermostability of the oligomerisation domain; and/or
4. (iv) they may modulate (for example improve) the solubility of the oligomerisation domain; and/or
5. (v) they may modulate (for example improve) the *in vivo* half-life of the oligomerisation domain.

[0037] In the context of a modified oligomerisation domain, the term "mutation" is as defined above.

[0038] In all cases, the mutations may modulate an immunological/antigenic property of a CBM or oligomerisation domain or a sialic acid binding molecule comprising the same.

[0039] There are a number of factors that influence the immunogenicity of a protein. One key determinant is the presence of intrinsic epitopes that can trigger T-cell dependent events that lead to antibody production. T-cells recognize short peptide fragments derived from the processing of the protein antigen that occurs within an antigen-presenting cell (APC). Some of these fragments will form complexes with human leukocyte antigen (HLA)/major histocompatability complex (MHC) class II molecules, and, if bound stably enough, will be carried to the surface of the APC and presented to T-cells. The recognition of non-self peptide by the T-cell receptor triggers the subsequent immune response. As such, in order to provide CBMs which are less immunogenic, wild type CBM and/or oligomerisation domains may be analysed in order to identify those regions which are immunogenic/antigenic (i.e. regions or domains which are likely to harbour or contain immunological epitopes). Immunogenicity (or antigenicity) is a property that may be assessed relative to, for example a particular human or animal host - in other words a CBM and/or oligomerisation domain may be analysed in order to determine which regions or domains may be immunogenic/antigenic in a specific (for example human) host. Immunogenicity (or antigenicity) may be assessed using, for example *in silico* in screening techniques (screening for, for example, T cell epitopes: including (as an example) ProPred *in silico* analysis), T-cell proliferation assays (including, Proimmune Human donor T-cell proliferation assay) and other immunological techniques. Further information regarding methods to "deimmunize" a protein may be derived from Jawa V, et al. ((2013) T-cell dependent immunogenicity of protein therapeutics: Preclinical assessment and mitigation. Clin Immunol. 149, 534-555) and Singh H, and Raghava GP ((2001) ProPred: prediction of HLA-DR binding sites. Bioinformatics. 17(12), 1236-7).

[0040] Using these techniques, it has been possible to identify immunogenic/antigenic regions of both the CBM and oligomerisation domains. Figure 1 shows a complete analysis (ProPred predictions) of the antigenic regions within a SpCBM sequence (Figure 1A) and a PaTD sequence (Figure 1B). Predicted binders are coloured blue,

with the first residue of each binding region shown in red. Predicted antigenic peptides (green bars) and ProImmune (purple bars) are shown under the sequences.

[0041] For example, within the SpCBM molecule, various regions between residues 127 to 300 (as shown in Figure 1A) have been identified as harbouring immunogenic/antigenic domains. For example regions spanning residues 127-135, 146-154, 154-166, 158-166, 143-151, 149-157, 167-176, 167-178, 182-196, 188-196, 185-193, 204-212, 213-221, 220-228, 239-246, 239-254, 241-249, 242-249, 241-250, 241-251, 239-251, 239-249, 239-247, 246-254, 243-251, 257-265, 267-275, 284-300, 284-299, 284-297, 286-284, 286-300 and 289-296 have been identified as potentially immunogenic (some of which are predicted to bind a small number of alleles, whereas others are regarded as moderately or highly immunogenic).

[0042] More specifically and using the above described *in silico* and immunologic (T cell proliferation) assays) the regions spanning residues:

167 to 178 of SEQ ID NO: 1 (i.e. sequence: FYNLFSVSSATK)

167 to 181 of SEQ ID NO: 1 (i.e. sequence: FYNLFSVSSATKKDE)

239 to 251 of SEQ ID NO: 1 (i.e. sequence: VRLYVNGVLSRTS)

236 to 250 of SEQ ID NO: 1 (i.e. sequence KGRVRLYVNGVLSRT)

245 to 254 of SEQ ID NO: 1 (i.e. sequence: GVLSRTSLRS)

286 to 294 of SEQ ID NO: 1 (i.e. sequence IRNLTVYNR)

have been identified as immunogenic/antigenic. It should be noted that the region spanning residues 245 to 254 is identified representing an area of significant immunogenicity.

[0043] Within the PaTD oligomerisation domain, regions between residues 336 to 424 has been identified as harbouring immunogenic/antigenic domains. For example, the regions spanning residues 336-344, 340-348, 341-349, 348-356, 353-363, 355-363, 355-370, 362-370, 362-370/371, 375-383, 375-390, 400-407, 402-410, 397-405, 403-410 and 416-424 have been identified as potentially immunogenic (some of which are predicted to bind a small number of alleles, whereas others are regarded as moderately or highly immunogenic). By way of non-limiting example, residues 338 to 352 of SEQ ID NO: 13 have been identified as harbouring immunogenic/antigenic domains.

[0044] More specifically and using the above described *in silico* and immunologic (T cell proliferation) assays) the regions spanning residues:

340 to 349 of SEQ ID NO: 13 (i.e. sequence: WFSVSSNSLY)

351 to 359 of SEQ ID NO: 13 (i.e. sequence: LSHGLQRSP)

398 to 406 of SEQ ID NO: 13 (i.e. sequence: GSLNIRLGT)

392 to 406 of SEQ ID NO: 13 (i.e. sequence: GAQVEVGSLNIRLGT)

338 to 352 of SEQ ID NO: 13 (i.e. sequence: SDWFSVSSNSLYTLS)

have been identified as immunogenic/antigenic. It should be noted that the regions spanning residues 340-349, 351-359 and 398-406 were identified representing areas of significant immunogenicity.

[0045] Collectively, these regions (from both the CBM and oligomerisation domain) will be referred to as regions of "immunogenicity/antigenicity".

[0046] Accordingly, a modified CBM and/or oligomerisation for use can be generated by mutating (for example

substituting, deleting, adding to, inverting and/or duplicating) one or more of the amino acid residues (or amino acid sequences) within, for example, the above identified regions of immunogenicity/antigenicity.

[0047] Thus, a modified CBM or oligomerisation domain according to this disclosure may comprise a modified immunogenicity (or antigenicity) profile. In other words, as compared to a wild type CBM or oligomerisation domain, a modified CBM/oligomerisation domain according to this disclosure may be less (or differently) immunogenic/antigenic in a human or animal host. One of skill will appreciate that mutations introduced to the primary amino acid sequence of a CBM or an oligomerisation domain, can modulate immunogenicity by rendering certain epitopes more or less immunogenic.

[0048] Thus, in one example, the disclosure provides a method of obtaining a CBM with a modified immunologic profile, said method comprising, determining which regions or domains of a CBM primary, secondary and/or tertiary amino acid sequence/structure are immunogenic and mutating one or more of the amino acid residues or sequences within one or more of the determined immunogenic/antigenic regions or domains.

[0049] Further, the disclosureprovides a modified CBM obtainable by a method of obtaining a CBM with a modified immunologic profile (as described above).

[0050] Further, the disclosure provides a method of obtaining an oligomerisation domain with a modified immunologic profile, said method comprising, determining which regions or domains of an oligomerisation domain CBM primary, secondary and/or tertiary amino acid sequence/structure are immunogenic and mutating one or more of the amino acid residues or sequences within one or more of the determined immunogenic/antigenic regions or domains.

[0051] Further, the disclosure provides a modified oligomerisation domain obtainable by a method of obtaining an oligomerisation domain with a modified immunologic profile (as described above).

[0052] The disclosure further provides a method of obtaining a modified multimeric CBM. The method may comprise:

obtaining at least one CBM with a modified immunologic profile as set out above; and optionally obtaining an oligomerisation domain with a modified immunologic profile as set our above; and

creating a multimeric CBM which comprises at least one modified CBM.

[0053] CBM/oligomerisation domain immunogenicity/antigenicity may be subject to individual amino acid mutations. For example a specific amino acid residue may be replaced (substituted with another) or deleted. In order to predict the effect of any given mutation, a modified sequence (the sequence containing at least one mutation verses a reference sequence) may be fed into, for example, a program designed to identify immunogenic sequences (including for example MHC binding regions within an antigen sequence). One of skill will be familiar with suitable programs and systems - but ProPred is one example: the aim of this server is to predict MHC Class-II binding regions in an antigen sequence, using quantitative matrices derived from published literature by Sturniolo *et. al.,* 1999. The server will assist in locating promiscuous binding regions.

[0054] The user will aim to identify those mutations that might have an effect on the immunogenicity/antigenicity of the CBM/oligomerisation domain but at the same time not affect the protein structure (which may be crucial to the sialic acid binding property of the CBM and to the oligomerisation property of the oligomerisation domain). For example, the modified CBM disclosed herein may maintain binding affinity for sialyllactose as assessed by, for example, surface plasmon resonance (SPR).

[0055] Based on the above and using the SpCBM molecule as an example, one or more of the following residues may be mutated:

Residue(s) 167 (F), 168 (Y) , 169 (N), 170 (L), 171 (F), 172 (S), 173 (V), 174 (S), 175 (S), 176 (A), 177 (T), 178 (K), 179 (K), 180 (D), 181 (E), 236 (K), 237 (G), 238 (R), 239 (V), 240 (R), 241 (L), 242 (Y), 243 (V), 244 (N), 245 (G), 246 (V), 247 (L), 248 (S), 249 (R), 250 (T), 251 (S), 252 (L), 253 (R), 254 (S), 286 (I), 287 (R), 288 (N), 289 (L), 290 (T), 291 (V), 292 (Y), 293 (N) and/or 294 (R)

[0056] Based on the above and using the PaTD as an example, one or more of the following residues may be mutated:
Residue(s) 338 (S), 339 (D), 340 (W), 341 (F), 342 (S), 343 (V), 344 (S), 345 (S), 346 (N), 347 (S), 348 (L), 349 (Y), 350 (T), 351 (L), 352 (S), 353 (H), 354 (G), 355 (L), 356 (Q), 357 (R), 358 (S), 359 (P), 392 (G), 393 (A), 394 (Q), 395 (V), 396 (E), 397 (V), 398 (G), 399 (S), 400 (L), 401 (N), 402 (I), 403 (R), 404 (L), 405 (G) and/or 406 (T).

[0057] It should be noted, that the term "mutation" includes the addition of further amino acids(s) to any of the regions (the "immunogenicity/antigenicity regions) containing these residues. Further, the term "mutation" may include the duplication of any one or more amino acids and/or the inversion of any sequence within these regions. For example, short sequences of two or more (for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 (or more)) amino acids may be inverted (and/or duplicated). Where the mutation is a substitution, the substituted amino acid may be any one of the other 19 naturally occurring amino acids or an artificial or synthetic amino acid. Further, the substituted amino acid may be a derivative or analogue of the wild type amino acid at the relevant position.

[0058] By way of non-limiting example and with reference to SpCBM, one or more the following mutations may be made (note, not all of these mutations may be directed at modulating the immunogenicity of a CBM) - for example, one or more of the mutations (including mutations M156F and M185I) may be used to modulate thermostability:

(i)	M156F	
(ii)	Y168W	(designated "lm15")
(iii)	L170A	(designated "lm16")
(iv)	L170T	(designated "lm17")
(v)	V173G	(designated "lm18")
(vi)	M185I	
(vii)	V239A	(designated "lm19")
(viii)	V239T	(designated "lm20")
(ix)	V246G	(designated "lm21")
(x)	I286A	(designated "lm22")
(xi)	Y292E	(designated "lm23")

[0059] Further, and again by way of non-limiting example and with reference to the PaTD molecule, one or more of the following mutations may be made:

(i)	S342D	(designated "lm24")
(ii)	S345D	(designated "lm25")
(iii)	L348D	(designated "lm26")
(iv)	R403K	(designated "lm27")

[0060] For the avoidance of doubt, any of CBM mutations listed as (i)-(xi) above and any of the PaTD mutations listed as (i)-(iv) may be made individually and/or in combination with one or more other of the listed mutations. For example, mutations at two or more of residues 156, 170 and 185 or 239, 246, 286 and 292 or 156, 170, 185, 239, 246, 286 and 292 may be combined. Additionally, or alternatively, mutations at residues 239 and 246 or 286 and 292 may be combined.

[0061] Further, the mutations listed above are examples only and it should be understood that any mutation at the noted positions which results in a CBM/oligomerisation domain having the desired properties (for example, reduced immunogenicity/antigenicity in a human host) is to be included within the scope of this disclosure.

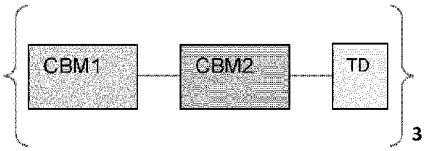
[0062] Many techniques exist to allow one of skill in this field to introduce one or more amino acid mutations into a CBM or oligomerisation domain sequence. Any of those techniques may be used here and they include, for example, mutagenesis techniques, including: (site-) directed mutagenesis, PCR mutagenesis, insertional mutagenesis, signature tagged mutagenesis, transposon mutagenesis and/or sequence saturation mutagenesis. Gene synthesis techniques may also be used to introduce any one or more of the mutations described herein.

[0063] Prior to any mutagenesis procedures, a wild type CBM and/or oligomerisation domain nucleic acid sequence may be subject to a codon-optimisation process in which the codons are optimised for expression in an expression system, such as, for example, a bacterial (e.g. an *E.coli*) expression system.

[0064] While useful sialic acid binding molecules may comprise a monomeric modified CBM (i.e. a single modified CBM), others may be multimeric - in other words, molecules which comprise two or more CBMs (for example, two or more modified CBMs). In such cases, the sialic acid binding molecule may be referred to as a "multimeric CBM". Sialic acid binding molecules, which comprise a plurality of CBMs, may comprise two or more modified CBMs wherein some but not all of the CBMs within the sialic acid binding molecule are modified. In other words, a sialic acid binding molecule, which comprises two or more CBMs, may comprise a mixture of modified and non-modified CBMs.

[0065] In one example, the sialic acid binding molecules may comprise hexameric CBMs. A hexameric CBM comprises six CBM monomers. In most cases, a hexameric CBM comprises two fused CBMs which are further conjugated to themselves via a fused oligomerisation (trimerisation) domain.

[0066] By way of example, the generic structure of a useful hexameric CBM molecules may be represented as:



[0067] The schematic (and generic) structure above shows a sialic acid binding molecule comprising three repeat units each comprising 2 CBMs (CBM1 and CBM 2) and an oligomerisation domain (in this case, a trimerisation domain: TD).

[0068] As described herein, one or both of the CBM moieties may be a modified CBM as described herein. Each repeat unit may be the same or different - in other words, while each of the repeat units may comprise 2 CBMs (modified or otherwise). The type of CBM and/or the level or degree of modification, may vary. By way of non-limiting example, one repeat unit may contain two CBMs with wild type sequences. Another may comprise two different CBMs one with a wild type sequence and one with a modified sequence (comprising one or more mutations as described herein). The third repeat unit may comprise two modified CBMs (comprising one or more of the mutations described herein). One of skill will appreciate that other combinations of CBM type and/or sequence (wild type vs mutated sequence) may be prepared and tested.

[0069] By way of non-limiting example, the following represent individual units (referred to as "HEX" units) which may be used to make hexameric sialic acid binding molecules. In each case, the HEX unit comprises two modified CBMs (denoted CBM1 and CBM 2) with the specific mutations introduced to each CBM being identified in parenthesis. It should be noted that a "----" symbol indicates an amino acid linker (linking one CBM to another or a CBM to an oligomerisation domain). As such, a hexameric sialic acid binding molecule may be made up of several (for example 3) HEX units. In each case, the oligomerisation domain (denoted "TD") conjugates the units together

as a trimer. While any given hexamer may comprise identical copies of the units described above (and below) under the headings HEX1, HEX2, HEX3, HEX4, HEX5, HEX6 and HEX17, one of skill will appreciate that further options are available. For example, a HEX unit may be made up of two CBMs, each having different mutations (the mutations being one or more selected from the options detailed herein).

1. **(i) HEX1**
   CBM1 (L170T V239A V246G I286A Y292E)-----**CBM2** (L170T V239A V246G I286A Y292E)-----**TD** (S342D L348D R403K)
2. **(ii) HEX2**
   CBM1 (V239A V246G I286A Y292E)----**CBM2** (V239A V246G I286A Y292E)----**TD** (S342D R403K)
3. **(iii) HEX3**
   CBM1 (V239A V246G I286A)-----**CBM2** (V239A V246G I286A)-----**TD** (S342D R403K)
4. **(iv) HEX4**
   CBM1 (V239A V246G)-----**CBM2** (V239A V246G)-----**TD** (S342D)
5. **(v) HEX5**
   CBM1 (V239A V246G)-----**CBM2** (V239A V246G)-----**TD** (R403K)
6. **(vi) HEX6**
   CBM1 (V239A V246G)-----**CBM2** (V239A V246G)-----**TD** (S342D R403K)
7. **(vii) HEX17**
   CBM1 (V239A V246G A162P)-----**CBM2** (V239A V246G A162P)-----**TD** (S342D R403K)

[0070] It will be noted that HEX6 and HEX17 are identical except for the additional A162P mutation. This proline mutation (a substitution for the wild type Alanine at residue 162) has been shown to improve thermostability (the single CBM Tm by 3-4°C). Further information regarding the use of proline mutations may be derived from Fu 2009, 'Increasing protein stability by improving beta-turns' (DOI 10.1002/prot.22509) describes the general approach. The proline mutation does not affect (increase or decrease) the predicted immunogenicity of the CBM molecule, is not located near the other mutations, the N- or C-termini or the ligand binding site. Rather unexpectedly, beyond the modest improvement in thermostability, it was noted that the A162P mutation yields hexameric CBMs (i.e. HEX17) exhibiting a marked improvement in *in vivo* experiments - in particular in comparison to those same experiments conducted using HEX6. For example, the modified molecules (in particular a molecule comprising 3 x HEX17 units) exhibit modulation over pro-inflammatory cytokines, including for example IL-8. Indeed, the modulatory effect (specifically an inhibitory effect) on the production of IL-8 by a molecule comprising 3 x HEX17 units, was improved over other tested modified molecules.

[0071] Relative to the amino acid sequences of Sp2CBMTD (aka "SpOrig") the amino acid sequence of the HEX6 and HEX 17 molecules is:

SpOrig	GAMVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDAKAPAFYNLFSVSSAT
HEX6	GAMVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDAKAPAFYNLFSVSSAT
HEX17	GAMVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDPKAPAFYNLFSVSSAT
SpOrig	KKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTVEKPTAELPKG
HEX6	KKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTVEKPTAELPKG
HEX17	KKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTVEKPTAELPKG
SpOrig	RVRLYVNGVLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIRNLTVYNRALT
HEX6	RARLYVNGGLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIRNLTVYNRALT
HEX17	RARLYVNGGLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIRNLTVYNRALT
SpOrig	PEEVQKRSGGGSGVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDAKAPAF
HEX6	PEEVQKRSGGGSGVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDAKAPAF
HEX17	PEEVQKRSGGGSGVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDPKAPAF
SpOrig	YNLFSVSSATKKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTV

HEX6	YNLFSVSSATKKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTV
HEX17	YNLFSVSSATKKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTV
SpOrig	EKPTAELPKGRVRLYVNGVLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIR
HEX6	EKPTAELPKGRARLYVNGGLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIR
HEX17	EKPTAELPKGRARLYVNGGLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIR
SpOrig	NLTVYNRALTPEEVQKRSGGALGVPDFESDWFSVSSNSLYTLSHGLQRSPRRVVVEFARS
HEX6	NLTVYNRALTPEEVQKRSGGSLGVPDFESDWFDVSSNSLYTLSHGLQRSPRRVVVEFARS
HEX17	NLTVYNRALTPEEVQKRSGGSLGVPDFESDWFDVSSNSLYTLSHGLQRSPRRVVVEFARS
SpOrig	SSPSTWNIVMPSYFNDGGHKGSGAQVEVGSLNIRLGTGAAVWGTGYFGGIDNSATTRFAT
HEX6	SSPSTWNIVMPSYFNDGGHKGSGAQVEVGSLNIKLGTGAAVWGTGYFGGIDNSATTRFAT
HEX17	SSPSTWNIVMPSYFNDGGHKGSGAQVEVGSLNIKLGTGAAVWGTGYFGGIDNSATTRFAT
SpOrig	GYYRVRAWI
HEX6	GYYRVRAWI
HEX17	GYYRVRAWI

[0072] Without wishing to be bound by any particular use or application, the disclosed molecules may be useful:

(i) in the treatment and/or prevention of diseases in a subject in need thereof;

(ii) in modulating immune responses (in human or animal subjects);

(iii) in modulating cell growth, proliferation and/or differentiation

(iv) as adjuvants; and/or

(vi) in the manufacture of medicaments.

[0073] Again, without wishing to be bound by any specific use, the disclosed molecules may be used (as medicaments) in the treatment and/or prevention of a variety of diseases and or conditions - in particular, diseases or conditions caused or contributed to by a pathogen, particularly those pathogens which are capable of binding cell surface sialic acid-containing receptors. The present disclosure may also provide methods useful in the treatment of subjects, particularly human subjects, suffering from, or at risk of contracting, a disease and/or condition caused or contributed to by a pathogen, particularly those pathogens which are capable of binding cell surface sialic acid-containing receptors. For example, the term "pathogen" may encompass any pathogen which is capable of binding, recognising or otherwise associating with a cell surface carbohydrate; especially those pathogens which have evolved to exploit or utilise the presence of cell surface (sialic acid) carbohydrates as a means of binding/adhering to and/or entering a cell. As such, the term "pathogen" may embrace microbial pathogens and may include respiratory pathogens such as viruses belonging to the Orthomyxoviridae or Paramyxoviridae families, for example, influenza and human parainfluenza, as well as bacteria belonging to the Streptococcus genus (such as *Streptococcus pneumoniae*) and/or *Haemophilus influenzae, Pseudomonas aeruginosa* - all of which are capable of binding carbohydrates on the surface of mammalian cells. One of skill will appreciate that the mammalian cell most frequently colonised/infected by pathogens of the type described herein, are epithelial cells, especially those lining the mucosal tracts (for example, respiratory epithelial cells). For a full disclosure of how sialic acid binding molecules can be used to treat or prevent disease, see PCT/GB2009/002189.

[0074] In terms of modulating immune responses (in human or animal subjects), the various sialic acid binding molecules described herein (which molecules comprise one or more modified CBMs) have immunomodulatory properties. For example (and without wishing to be bound by theory), the disclosed sialic acid binding molecules

modulate host immune responses, prime the immune system, modulate (e.g. increase or decrease) the expression, function and/or activity of immune system processes, pathways and/or any component(s) thereof. The term "priming" as applied to an immune response, may encompass the phenomenon of increasing the readiness and/or responsiveness of an immune system to an immunogen, antigen, pathogen, disease or infection. Without wishing to be bound by theory, in addition to any immunomodulatory properties associated with the sialic acid binding molecules disclosed herein, subjects administered or contacted with the disclosed sialic acid binding molecules may be rendered better able to cope with the onset of an infection/disease. For a full disclosure of the immunomodulatory activity of sialic acid binding molecules see PCT/GB2015/050161.

[0075] Further, it has been shown that the disclosed sialic acid binding molecules (which molecules may contain one or more modified CBMs) may be used to modulate aspects of cell growth and/or cell activity. The terms "growth" and "activity" as applied to cells may embrace processes and/or phenomena associated with one or more of cell proliferation, cell viability, cell migration, cell metabolism, cell differentiation and/or cell morphology/phenotype. The terms "growth" and/or "activity" may further include the response of a cell to certain exogenous and/or endogenous factors or stimuli including, for example, responses to certain compounds of the immune system, cytokines, chemokines and one or more environmental factors (light, temperature, pressure, mechanical stress and the like). Thus, the sialic acid binding molecules disclosed herein may be used to modulate (inhibit, decrease or increase) levels of cell responsiveness. Accordingly, any of the disclosed molecules may be used in the treatment or prevention of a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or activity or in the manufacture of a medicament to treat or prevent the same.

[0076] Without wishing to be bound to any particular medical application, it should be noted that diseases which are caused, contributed to or characterised by aberrant cell growth and/or activity may include, for example cell proliferation and/or differentiation disorders including, those referred to or classified as benign or malignant conditions. For example, the term "cell proliferation and/or differentiation disorders" may include those diseases and/or conditions collectively referred to as "cancer". The term "cancer" may include, but is not limited to, those cancers referred to as forms of breast cancer, bone cancer, brain cancer (gliomas), pancreatic cancer, lung cancer, prostate cancer, skin cancer, ovarian cancer, cervical cancer, head and neck cancers and bowel/colon cancer. The term "cancer" may also include those diseases and/or conditions collectively referred to as "leukaemias" (both chronic and acute) and any cancer affecting a mucosal/mucosal associated surface or tissue.

[0077] As such, a sialic acid binding molecule described herein, which molecule may comprise one or more modified CBMs, may find application (i.e. may be for use) in the treatment and/or prevention of cancer. The disclosed sialic acid binding molecules may further be used in the manufacture of a medicament for the treatment and/or prevention of cancer. For a full disclosure of how sialic acid binding molecules can be used to modulate cell growth and/or cell activity, see PCT/GB2017/052808.

[0078] Any of the disclosed sialic acid binding molecules (comprising one or more modified CBMs) may be used as adjuvants which themselves may be used in combination with one or more antigens to augment, modulate or enhance a host immune response to the one or more antigens. It should be noted that while the sialic acid binding molecule-based adjuvants disclosed herein can be combined with any type of antigen, the adjuvants, which are the subject of this disclosure may be particularly useful as mucosal adjuvants - in other words, for use with antigens that are to be administered mucosally. For a full disclosure of how sialic acid binding molecules can be used as adjuvants, see PCT/GB2017/052805.

[0079] It has also been shown that the disclosed sialic acid binding molecules (which molecules may contain one or more modified CBMs) may find utility in the treatment and/or prevention of sepsis. The term "sepsis" is applied to a number of diseases, conditions and/or syndromes which may have an infectious (for example viral, bacterial and/or fungal) aetiology. For example, term "sepsis", may embrace those disease states, conditions or syndromes referred to as SIRS (systemic inflammatory response syndrome: see Singer et al, 2016: JAMA "The third International consensus definitions for sepsis and septic shock), sepsis (which is often defined as "SIRS in response to an infectious process"), severe sepsis (that is sepsis with sepsis-induced organ dysfunction or tissue hypoperfusion (which itself might manifest as hypotension, elevated lactate or decreased urine output) and septic

shock (severe sepsis plus persistently low blood pressure despite, for example, the administration of intravenous fluids). The term "sepsis" is most often applied to diseases, conditions and/or syndromes which result from "bacterial sepsis". Bacterial sepsis may stem from the presence of bacteria in blood and may sometimes be referred to as "bacteraemia" or "septicaemia". The term "sepsis" may also embrace diseases and/or conditions which are caused or contributed to by the presence of bacterial components such as LPS, toxins and/or membrane fragments in the blood. Components of this type may originate from primary infections present in other tissues and/or organs, for example, infections present in the lungs, brain, skin, urinary tract, pelvis and/or abdomen.

[0080] For a full disclosure of how sialic acid binding molecules can be used in the treatment and/or prevention of sepsis, see PCT/GB2017/052800.

[0081] The modified CBMs described herein and any molecules comprising the same may be conjugated, bound or joined to or associated with, other entities for the purpose of targeting or delivering that entity to some tissue or cell. Molecules of this type may be otherwise known as "therapeutic warheads" or "conjugates". Without wishing to be bound by theory, the presence of ligands for the various modified CBMs which may be comprised within the molecules of this disclosure, in certain cell receptors and membrane bound molecules, may allow the various molecules described herein to be exploited as a means to deliver conjugated heterologous molecules (these being molecules which are distinct from and different to the modified CBM or molecule comprising the same) to said cells or tissues comprising said cells. Such conjugated molecules may be useful in the treatment of a variety of diseases including, for example, cancer, where the conjugated molecules described herein (which molecules exhibit affinity for carbohydrates expressed on the surface of cells) may be used to direct therapeutic and/or cytotoxic moieties thereto.

[0082] By way of example, a molecule as described herein (including any of the modified CBMs or molecules comprising the same) may be conjugated to one or more (for example two, three, four or more) moieties which are, for example, therapeutic and/or cytotoxic.

[0083] Useful molecules may comprise a modified CBM of this disclosure conjugated (joined, bound or otherwise associated with) to a heterologous moiety. The heterologous moiety may comprise a therapeutic and/or cytotoxic moiety which may be conjugated to some part of the modified CBM molecule.

[0084] For example, the heterologous moiety may be conjugated to one or both ends of a modified CBM molecule. The heterologous moiety may be additionally or alternatively conjugated (or even fused) to an internal portion of a modified CBM molecule. It will be appreciated that however the heterologous moiety is to be conjugated to the modified CBM molecule, the molecule (nor its conjugation) should not (substantially) interfere with or ablate or reduce the carbohydrate (sialic acid) binding property of the modified CBM molecule.

[0085] As stated, the heterologous moiety may be a drug useful in the treatment of a disease which affects a cell or tissue expressing a receptor which comprises the ligand for any one of the modified CBMs (or molecules comprising the same) described herein. For example, the drug may be a chemotherapeutic drug for use in the treatment of cancer and the like. The heterologous moiety may be a cytotoxic moiety capable of killing or inducing apoptosis in, a cell. The heterologous moiety may comprise a molecule which is able to recruit specific cells to or into a particular tissue. For example, the heterologous moiety may be, for example, a T cell receptor (TCR) which may be used as a means to recruit T cells to, for example a tumour or cancerous tissue.

[0086] The present disclosure may provide compositions for application in the various uses, medicaments and methods described herein. As such, any of the modified CBMs described herein (or any molecule comprising a modified CBM) may be formulated for use.

[0087] For convenience, and with reference to the section below describing compositions, formulations and the like, it should be noted that both molecules comprising a modified CBM as described herein, a molecule comprising the same and any conjugates comprising the same (for example modified CBM :: drug

conjugates/fusions) shall be included under the general term "molecule comprising a modified CBM".

[0088] A molecule comprising a modified CBM may be formulated for use as a therapeutic or pharmaceutical composition. The various compositions may comprise one or more of the molecules described herein and any given treatment may require the administration (together, concurrently or separately) of one or more of these compositions. It should be noted that a composition according to this disclosure may further comprise one or more other therapeutic moieties - for example molecules, small molecules, antibodies, oligonucleotides and the like useful in the treatment of one or more diseases and/or conditions. Additionally, or alternatively, the modified CBMs may be administered together with one or more other (different) therapeutic entities - wherein the one or more other (different) therapeutic entities may be used for the treatment of the same or a different disease. The term "administered together" embraces administration of a modified CBM before, after and/or at the same time as the administration of the one or more other therapeutic entities.

[0089] The molecules described herein may be formulated for enteral (including oral), parenteral and/or topical administration and one of skill will appreciate that the precise formulation may vary depending on the route of administration.

[0090] Pharmaceutical compositions according to the present invention may be prepared conventionally, comprising substances that are customarily used in pharmaceuticals and as described in, for example, Remington's The Sciences and Practice of Pharmacy, 22nd Edition (Pharmaceutical Press 2012) and/or Handbook of Pharmaceutical Excipients, 7th edition (compiled by Rowe et al, Pharmaceutical Press, 2012).

[0091] A therapeutic or pharmaceutical composition of this disclosure (that is a composition comprising a molecule comprising a modified CBM and for use in any of the medicaments or methods described herein) may be formulated together with one or more pharmaceutically acceptable excipients, carriers, adjuvants and buffers. The compositions can be administered, e.g. orally (including mucosally), parentally, enterally, intramuscularly, subcutaneously, intravenously or via any other routes useful to achieve the desired effect (for example the modulation of cell growth/activity, treatment or prevention of diseases/conditions associated with the same and/or cancer and/or modulation of tumour growth). As stated, depending on the chosen route of administration, the exact composition of the formulation may vary.

[0092] A therapeutic or pharmaceutical formulation comprising a molecule comprising a modified CBM and for administration to a subject may be coated, encapsulated or enveloped in a material which protects the molecule from the action of enzymes, acids and other natural compounds/conditions (including, for example, compounds (including antibodies), cells and processes of the immune system) which may inactivate or denature the compound and/or its carbohydrate binding properties.

[0093] Among the various standard and conventional excipients that may be available for use in compositions comprising the molecules described herein, are those pharmaceutically acceptable organic or inorganic carrier substances which are suitable for parenteral, enteral, oral (including mucosal) and other routes of administration that do not deleteriously react with the molecule(s) comprising a modified CBM.

[0094] Where molecules comprising a modified CBM are to be formulated for parental administration, the compositions may be sterile.

[0095] The composition may comprise an oil-based or aqueous solution, a suspension and/or an emulsion.

[0096] In other embodiments, the composition may take the form of an implant, such as for example a (dissolvable or biodegradable) film, pessary or implant (including suppositories).

[0097] The pharmaceutical preparations comprising the molecules described herein may be mixed with stabilizers, wetting agents, emulsifiers, salts (for use in influencing osmotic pressure), buffers and/or other substances that do not react deleteriously with the active compounds.

[0098] One or more of the molecules described herein may be formulated for and administered, orally. As stated, oral administration would include mucosal administration which would itself would include administration intranasally and/or by inhalation.

[0099] Compositions for use may include solid dosage forms which are suitable for oral administration. These may include, for example capsules, tablets, pills, powders, and granules. In any given solid dosage form, a molecule comprising a modified CBM (or any molecule or conjugate comprising the same) may be admixed with at least one inert pharmaceutically-acceptable excipient. Examples of suitable excipients will be known to one of skill in this field but may include, for example fillers or extenders, humectants, wetting agents, binders, disintegrating agents, solution retarders, absorption accelerators, adsorbents, lubricants or mixtures thereof. A tablet, pill or capsule may further comprise a buffering agent. Solid dosage forms such as tablets, dragees, capsules, pills and/or granules also can be prepared with coatings and shells, such as coatings which protect against the gastrointestinal environment and/or stomach acid.

[0100] A solid dosage form may contain opacifying agents, and can also be formulated so as to ensure the delayed release of the active agent (in this case a molecule comprising a modified CBM or a conjugate comprising the same) in or to a specific part of the intestinal tract.

[0101] Solid compositions for oral administration can be formulated in a unit dosage form, each dosage containing an appropriate dose of a molecule comprising a modified CBM (or conjugate comprising the same). The exact amount of a molecule comprising a modified CBM (or conjugate comprising the same) contained within any given solid dosage form will vary depending on the intended use. A solid composition may contain a "unit dose" - a unit dose containing a quantity of a molecule comprising a modified CBM (or conjugate containing the same) calculated to produce the desired effect (for example modulation of cell growth and/or activity) over the course of a treatment period.

[0102] Liquid dosage forms for oral administration may (as stated) include emulsions, solutions, suspensions, syrups, and elixirs. In addition to the compound or composition, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers.

[0103] Any of the disclosed molecules may be used in any suitable amount. As stated, the molecules may be formulated for oral, mucosal or parenteral administration and as such, the precise formulation may depend on the intended route of administration and/or physiological and/or other attributes of the subject. The amount of a molecule comprising a modified CBM present in any given dose may be in the region of 0.1 µg -1000 µg. For example, amounts of about 0.1 µg, 0.2 µg, 0.3 µg, 0.4 µg, 0.5 µg, 1 µg, 10 µg, 20 µg, 25 µg, 50 µg, 100 µg, 200 µg, 300 µg, 400 µg, 500 µg, 600 µg, 700 µg, 800 µg or 900 µg. Higher amounts of modified CBM (or a molecule comprising the same) may be used; for example, amounts in the region of 1mg-10mg, for example amounts of about 1 mg, 2 mg, 3 mg, 4 µg, 5 mg, 6 mg, 7 mg, 8 mg or 9 mg. The selected amount of the modified CBM molecule may be formulated in a specific volume of a pharmaceutically acceptable excipient, diluent and/or buffer. The volume of excipient, diluent or buffer may be about 10 µl to 5 ml. For example, the required amount of CBM32/47/67/70 molecule may be combined (or formulated) with about 15 µl, 20 µl, 25 µl, 30 µl, 35 µl, 40 µl, 45 µl, 50 µl, 55 µl, 60 µl, 65 µl, 70 µl, 75 µl, 80 µl, 85 µl, 90 µl, 95 µl, 100 µl, 200 µl, 250 µl, 300 µl, 400 µl, 500 µl, 600 µl, 700 µl, 800 µl, 900 µl, 1 ml, 2 ml, 3 ml or 4ml. Doses at concentrations of about 0.1 µg/ml-10 mg/ml may be used including, for example, doses at 5 µg/ml, 10 µg/ml, 20 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 500 µg/ml, 600 µg/ml, 700 µg/ml, 800 µg/ml or 900 µg/ml, 950 µg/ml, 1 mg/ml, 1.5mg/ml, 2mg/ml, 2.5 mg/ml, 3mg/ml, 3.5mg/ml, 4mg/ml, 4.5mg/ml, 5mg/ml, 5.5mg/ml, 6mg/ml, 6.5mg/ml, 7mg/ml, 7.5mg/ml, 8mg/ml, 8.5 mg/ml, 9mg/ml or 9.5mg/ml.

[0104] In use, a dose of a modified CBM molecule, administered as part of the treatment and/or prevention of a disease (for example a cell proliferation and/or differentiation disorder or cancer), may be administered multiple times over a number of days, weeks months or years. For example, after an initial (or first) administration, a dose of a modified CBM molecule may be administered again at about (+/- 1 or 2 days) 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

14, 15, 16, 17, 21, 28 and/or 35 days later. Multiple repeat doses may be given at regular intervals - for example every 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28 day(s) after a preceding dose. On any given day, a specific dose of a modified CBM molecule may be administered 1, 2, 3 or more times. Each time, the modified CBM molecule may be administered (by whatever route is considered best) to affect a suitable treatment or to induce prophylaxis against a particular disease or condition.

DETAILED DESCRIPTION

[0105] The present disclosure will now be described in detail with reference to the following figures which show:

Figure 1: ProPred predictions of antigenic peptides. A. SpCBM sequence. B. PaTD sequence. Predicted binders are coloured blue, with the first residue of each binding region shown in red. Antigenic peptides predicted by Nordic Biopharma (green bars) and ProImmune (purple bars) are shown under the sequences.

Figure 2: Expression test of wild type and mutated domains. Lane 1, M12 standard; Lane 2, WTSpCBM; Lanes 3-11, Im15-Im23; Lanes 12-15, Im24-Im27; Lane 16, WT *PaTD.* A) Whole cell extracts, B) Soluble extracts.

Figure 3: Position of peptide 167-181 in the SpCBM structure

Figure 4: Expression and Ni-NTA pull-down of variants Im28 to Im34

Figure 5: Sites of the HEX17 mutations on the hexamer structure. The quarternary structure of HEX17 was modelled by assembling the crystal structures of the individual SpCBM (pdb code 4c1x) and PaTD (pdb code 2w38) into the hexamer (i.e. 6 copies of SpCBM and 3 copies of PaTD per molecule). The positions of the bound ligand (α2,3-sialyllactose) are shown in stick form (orange). The positions of the mutations are also shown: Blue, the sites of the A162P mutation; Cyan, the sites of the other two CBM mutations; Magenta, the sites of the TD mutations.

Figure 6: IL-8 stimulation. A549 cells were stimulated by the addition of 10 µg of biologic (Sp2CBMTD (aka SpOrig), HEX6 or HEX17). Cell supernatant was harvested at 24 h or 48 h timepoints and the IL-8 content was determined by ELISA. Statistical significance between control and treated cells was determined with one-way ANOVA using Tukey's multiple comparison test.

Figure 7: Multiplex analysis of inflammatory mediators. A549 cells were stimulated by the addition of 10 µg of biologic (Sp2CBMTD (aka SpOrig), HEX6 or HEX17). Cell supernatant was harvested at 6 h, 24 h or 48 h time-points and inflammatory mediators analysed using a Human Cytokine 12-plex Assay. Statistical significance between control and/or WT hexamer and hexamer variants was determined using a one-way ANOVA (Tukey's multiple comparison test).

Figure 8: Percentage survival of CBM-treated and untreated mice when lethally challenged with influenza strain PR8. CBM2, CBM3 and CBM4 represent HEX17, HEX6 and WT (SpOrig) respectively. Single CBM dosed animals were given 100 µg of CBM one day prior to lethal challenge with PR8; repeat dosed animal were given 2 x 0.1 µg of CBM at day-3 and day-1 prior to PR8 challenge.

Figure 9: Clinical scores of CBM-treated and untreated mice during PR8 infection. CBM2, CBM3 and CBM4 represent HEX17, HEX6 and WT (SpOrig) respectively. An ascending clinical score of 1 to 5 indicates no symptoms (1) to lethargy and death (5), respectively.

Figure 10: Percentage weight loss of CBM-treated and untreated mice during PR8 infection. CBM2, CBM3 and CBM4 represent HEX17, HEX6 and WT (SpOrig) respectively.

Figure 11: Anti-mCBM antibody analysis of Day 21 lung homogenates and sera tissue from a PR8-challenged mouse study. Statistical significance between WT hexamer and hexamer variants was determined using a one-way ANOVA (Tukey's multiple comparison test).

Figure 12a: Anti-CBM antibody levels from Day 35 BAL mouse samples.

Figure 12b: Anti-CBM antibody levels from Day 35 serum mouse samples

Methods and results

Sp2CBMTD: prediction of immunogenic regions

Nordic Biopharma *in silico* screen

[0106] The *in silico* T-cell epitope screening identified four significant and two borderline immunogenic clusters:
Significant:

Domain	Residue range	Sequence
*Sp*CBM	245 to 254	GVLSRTSLRS
*Pa*TD	340 to 349	WFSVSSNSLY
*Pa*TD	351 to 359	LSHGLQRSP
*Pa*TD	398 to 406	GSLNIRLGT

Borderline:

Domain	Residue range	Sequence
*Sp*CBM	167 to 178	FYNLFSVSSATK
*Sp*CBM	239 to 251	VRLYVNGVLSRTS

ProImmune Human donor T-cell proliferation assay

[0107] The ProImmune study highlighted two regions of high antigenicity and two regions of moderate antigenicity:
High antigenicity:

Domain	Residue range	Sequence
*Sp*CBM	236 to 250	KGRVRLYVNGVLSRT
*Pa*TD	392 to 406	GAQVEVGSLNIRLGT

Moderate antigenicity:

Domain	Residue range	Sequence
*Sp*CBM	167 to 181	FYNLFSVSSATKKDE
*Pa*TD	338 to 352	SDWFSVSSNSLYTLS

ProPred *in silico* analysis

[0108] A further *in silico* tool, the online ProPred server[4], was also used. The output of the ProPred server is shown in Figure 1. The relative positions of the Nordic Biopharma/ProImmune epitopes are also highlighted and

indicate reasonable agreement between the three methods. In addition to the epitopes listed above, ProPred strongly predicted another immunogenic epitope in the SpCBM domain:

Domain	Residue range	Sequence
*Sp*CBM	286 to 294	IRNLTVYNR

Mutations in the individual CBM and TD domains

[0109] To guide the design of mutations that might reduce immunogenicity, ProPred was used to test the effect of changing each residue in these peptides to every alternative residue. Those that gave the greatest reduction in predicted number of allele binders were noted. As the crystal structure of both the SpCBM and TD domains are known, these mutations were also modelled to reduce the likelihood of introducing mutations that would obviously disrupt the protein structure.

[0110] Initially, nine single mutations in SpCBM and four single mutations in PaTD were introduced and are listed below ('Im' is short for immunogenicity mutant):

(*Sp*CBM) variants	Mutation	(*Pa*TD) variants	Mutation
WT*Sp*	-	WTTD	-
Im15	Y168W	Im24	S342D
Im16	L170A	Im25	S345D
Im17	L170T	Im26	L348D
Im18	V173G	Im27	R403K
Im19	V239A		
Im20	V239T		
Im21	V246G		
Im22	I286A		
Im23	Y292E		
Note: Im1 to Im14 (not shown) were introduced by mutagenesis into a non-codon optimized background, before the ProImmune data were available.			

Synthesis of WT and mutated constructs

[0111] The genes encoding WT SpCBM, WT PaTD and the variants Im15 to Im27 were codon optimized for *E. coli* expression and synthesized by GeneArt. The genes were then cloned in-house into the pHISTEV vector for expression as 6His-tagged proteins.

Expression and biophysical characterization

[0112] An initial expression test was performed to assess solubility. The results show that all were expressed, but not all were soluble (Figure 2). Note: solubility (or a lack thereof) is not necessarily a predictor of utility. One of skill will appreciate that when manufacturing or producing proteins, certain processes require the use of insoluble material as this is readily purified (from inclusion bodies and the like). Downstream protocols may then re-engineer proteins to modulate features such as solubility.

[0113] Results of the expression test show that:

- Im16 (L170A) is insoluble or very poorly soluble
- Im25 (TD, S345D) is insoluble
- Im15 (Y168W) and Im17 (L170T) have reduced solubility
- Im18 (V173G) and Im22 (I286A) are slightly reduced.
- The remainder show soluble expression.

[0114] The 13 soluble proteins were expressed in *E. coli* and purified by immobilized metal affinity chromatography (IMAC), followed by TEV digestion to remove the 6His-tag, then reverse IMAC and size exclusion chromatography (SEC).

[0115] Ten purified domains (WTSp, Im19, Im20, Im21, Im22, Im23, WTTD, Im24, Im26 and Im27) were further characterized by:

1. (i) Thermofluor to measure melting temperature (Tm)
2. (ii) Near UV circular dichroism (CD) to compare tertiary structures to WT
3. (iii) Dynamic light scattering (DLS) to check oligomeric state in solution
4. (iv) Surface plasmon resonance (SPR) to measure binding affinity to sialyllactose
5. (v) Measurement of IL-8 cytokine stimulation

[0116] The results are summarized in Table 1.

Name	Mutation	Solubility	Purification	Tm +/- 6SL	NearUV CD	DLS	Biacore	Cytokine stimulation
WT*Sp*	-	Green	Green	Green	Green	Green	Green	Green
Im15	Y168W	Yellow	Orange	Red	N/A	N/A	N/A	N/A
Im16	L170A	Red	N/A	N/A	N/A	N/A	N/A	N/A
Im17	L170T	Yellow	Orange	N/A	N/A	N/A	N/A	N/A
Im18	V173G	Green'	Orange	N/A	N/A	N/A	N/A	N/A
Im19	V239A	Green	Green	Green	Green	Green	Green	N/D
Im20	V239T	Green	Green	Green'	Green'	Green	Green	N/D
Im21	V246G	Green	Green	Green'	Green	Green	Green	Green
Im22	I286A	Green'	Green	Green'	Green	Green	Green	Green
Im23	Y292E	Green	Green	Green'	Green'	Green	Yellow	Yellow
Im24	S342D	Green	Green	Green	Green	Green		
Im25	S345D	Red	N/A	N/A	N/A	N/A		
Im26	L348D	Green	Green	Green'	Green	Green		
Im27	R403K	Green	Green	Green	Green	Green		
WTTD	-	Green	Green	Green	Green	Green		

Table 1. Qualitative summary of the biophysical characterizations of the WT domains and their variants. Colour coding is from green to red (including green', orange and yellow), where green indicates that the variant closely resembles its WT counterpart for that particular characteristic and pale green (green') or yellow indicate increasing degrees of differences. Red or orange indicate significant differences. N/A: these characterizations were not performed due to poor solubility/purity of the protein. N/D: not determined.

***Sp* peptide 167-181:**

[0117] Im15, Im16, Im17, Im18 are all insoluble or poorly soluble (as stated, this does not necessarily impact on protein utility). These are in the 'moderately' antigenic region 167-181 (FYNLFSVSSATKKDE). This region is clearly very sensitive to change.

[0118] Earlier results show that M156F, which sits adjacent to L170 (and I286), increases Tm by ~4°C.

[0119] This could therefore be combined with L170T. M156F does not increase predicted immunogenicity.

[0120] M185I increases Tm by 5°C, and lies parallel to L170 (Figure 3). This mutation could also be included. Note that, like M156F, M185I does not increase predicted immunogenicity but slightly reduces the number of predicted allele binders.

***Sp* peptide 236-250:**

[0121] Im19, Im20, Im21 all behave similarly to WT. These are in the 'highly' antigenic region 236-250 (KGRVRLYVNGVLSRT).

[0122] Im19 (V239A) was chosen over the threonine mutation (Im20, V239T). There is no difference in predicted immunogenicity but Im19 is a closer match to WT Thermofluor Tm and Near UV spectrum. This would be combined with Im21 (V246G).

***Sp* peptide 286-294:**

[0123] Im22 (I286A) is broadly similar to WT while Im23 (Y292E) appears to exhibit reduced ligand affinity. This region, 286-294 IRNLTVYNR, was not flagged up by ProImmune but is strongly predicted by ProPred to be immunogenic.

[0124] There is some indication that Im22 has lower Tm than WT. This residue is adjacent to M156 so may behave differently if M156F was included.

TD peptide 338-352:

[0125] Im24 (S342D) and Im26 (L348D) show similar characteristics to the WT trimerization domain, but with some suggestion of reduced Tm in Im26. These are in the 'moderately' antigenic region 338-352 SDWFSVSSNSLYTLS. The WT sequence was predicted to bind 9 alleles, while Im24 predicts 2 alleles and a Im24/Im26 double mutant predicts 1 allele.

TD peptide 392-406:

[0126] Im27 (R403K) is similar to WT. It is part of the 'highly' antigenic region 392-406 GAQVEVGSLNIRLGT. Predicted alleles are reduced from 21 to 3 when this mutation is introduced.

Synthesis of multiple mutation combinations Im28-34

[0127] The following mutations were introduced:

1. i) M156F/L 170T
2. ii) M156F/L170T/M185I: In ProPred, alleles predicted for this region are reduced from 31 in the WT to 19 for this combination.
3. iii) V239A/V246G: In ProPred, alleles for this region are reduced from 44 to 3.

4. iv) I286A/Y292E: In ProPred, alleles are reduced from 41 to 1.
5. v) V239AA/246G/I286A/Y292E combines the previous two doubles.
6. vi) M156F/L170T/M185I/V239A/V246G/I286A/Y292E combines all the *Sp* mutations
7. vii) TD: S342D/L348D/R403K: Predicted alleles are reduced from 9 to 1 for TD peptide 338-352 and alleles for peptide TD peptide 392-406 are reduced from 21 to 3. This triple mutant combines all the TD mutants. They are all surface exposed and distal to the N-terminal end of TD, so would not be expected to interfere with SpCBM in the hexamer form.

[0128] The constructs are named Im28 to Im34:

(*Sp*CBM) variant	**Mutations**
Im28	M156F/L170T
Im29	M156F/L170T/M185I
Im30	V239A/V246G
Im31	I286A/Y292E
Im32	V239A/V246G/I286A/Y292E
Im33	M156F/L170T/M185I/V239A/V246G/I286A/Y292E
(*Pa*TD) variant	**Mutation**
Im34	S342D/L348D/R403K

2.5 Expression and biophysical characterization of Im28-Im34

[0129] As with the single mutations, the combinations Im28 to Im34 were synthesized by GeneArt and subcloned into pHISTEV for expression analysis. A nickel bead pull-down on the His-tagged soluble extract was also performed (Figure 4).

Hexameric forms

Design of hexameric constructs HEX1 to HEX17

[0130] Genes encoding the hexameric forms (called Hex1 to Hex17) were synthesized by GeneArt:

***Sp*2CBMTD**

variant	**Mutations**
HEX1	CBM1(L170T V239A V246G 1286A Y292E)-CBM2(L170T V239A V246G 1286A Y292E)-TD (S342D L348D R403K)
HEX2	CBM1(V239A V246G 1286A Y292E)-CBM2(V239A V246G 1286A Y292E)- TD (S342D R403K)
HEX3	CBM1(V239A V246G I286A)-CBM2(V239A V246G 1286A)-TD (S342D R403K)
HEX4	CBM1(V239A V246G)-CBM2(V239A V246G)-TD (S342D)
HEX5	CBM1(V239A V246G)-CBM2(V239A V246G)-TD(R403K)
HEX6	CBM1(V239A V246G)- CBM2(V239A V246G)-TD (S342D R403K)
HEX17	CBM1 (V239A V246G A162P) - CBM2 (V239A V246G A162P) -TD (S342D R403K)

[0131] The hexameric forms were synthesized in two parts to avoid problems associated with synthesising repeat sequences in the tandem CBM copies. The first gene covered the first CBM and the second part encompassed the second CBM plus the TD. These could then be simultaneously cloned into pHISTEV to create the Sp2CBMTD construct that trimerizes upon expression.

[0132] The first hexamer, HEX1, contained the mutations L170T/V239A/V246G/I286A/Y292E in the CBMs and S342D/L348D/R403K in the TD.

[0133] The solubility data of the individual domains indicated that HEX1 was unlikely to be soluble (again, not necessarily a reflection on the utility of the molecule); a further construct, HEX3, was synthesized. Note that HEX2 contained the same mutations as Hex3, but with the addition of Y292E.

[0134] HEX3 was synthesized and subcloned into the pHISTEV vector. Expression was insoluble under all conditions tested (varying temperature, IPTG concentration, cell density at induction, with or without heat shock). The CBM-only domain containing the same three mutations (V239A V246G I286A) is soluble. A double mutant (V239A V246G) behaves very similarly to WT. Therefore, further variants (HEX4, HEX5 and HEX6) were designed and constructed by PCR/ligations, which exclude I286A and contain either one or both of the TD mutations.

[0135] During the work on HEX6 a number of other versions were designed containing different combinations of the HEX6 mutations (numbered HEX7 to HEX16; not characterised).

[0136] HEX17 contains the HEX6 mutations with an additional A162P mutation. This proline mutation has been shown to increase the single CBM Tm by 3-4°C. The proline mutation is not near the other mutations, the N- or C-termini or the ligand binding site.

Characterization of the hexameric variants

[0137] The expression, purification and characterization results are shown in Table 2. Based on these results, HEX6 and HEX17 were taken forward. The positions of the HEX17 mutations on the hexamer are shown in Figure 5.

Table 2

Name	Mutations	Solubility	Purification	Thermostability	NearUV CD	Biacore	IL-8 assay
Hex1	L170T/V239A/V246G/I286A/Y292E/S342D/L348D/R403K	Red	N/A	N/A	N/A	N/A	N/A
Hex2	V239A/V246G/I286A/Y292F/S342D/R403K (designed but not made)						
Hex3	V239A/V246G/I286A/S342D/R403K	Red	N/A	N/A	N/A	N/A	N/A
Hex4	V239A/V246G/S342D	Yellow	Red	N/A	N/A	N/A	N/A
Hex5	V239A/V246G/R403K	Green	Yellow	Yellow	N/A	N/A	N/A
Hex6	V239A/V246G/S342D/R403K	Green	Green	Yellow	Green	Green	Yellow
Hex7 to 16	Note: These constructs are different combinations of the Hex6 mutations and were designed as a back-up in case Hex6 failed						
Hex17	A162P/V239A/V246G/S342D/R403K	Green	Green	Green	Green	Green	reduced IL-8

[0138] Table 2. Qualitative summary of the biophysical characterizations of the hexameric Sp2CBMTD variants. Colour coding is from green to red, where green indicates that the variant closely resembles its WT counterpart for that particular characteristic and pale green (green') or yellow indicate increasing degrees of differences. Red or orange indicate significant differences. N/A: these characterizations were not performed due to poor solubility/purity of the protein. N/D: not determined.

EXAMPLE 1: Inflammatory mediators.

Aim: To measure the innate immune response of mCBM-treated human lung epithelial cells (A549) by analysing levels of inflammatory mediators over time.

[0139] Administration of *Sp*2CBMTD to mammalian cells stimulated a pro-inflammatory response both *in vitro* and *in vivo*[1,2]. To determine whether this was still observed with modified hexameric sialic acid binding molecules, mammalian A549 cells were stimulated by the addition of 10 µg of biologic (*Sp*2CBMTD (aka SpOrig), HEX6 or HEX17 and cell culture medium was harvested at specific time-points post administration. The concentrations of inflammatory mediators were measured both by ELISA and a multiplex assay.

[0140] Human IL-8 (benchmark cytokine for the study) response using a human 1 × Mouse CXCL1/KC Quantikine ELISA Kit (R&D BioSystems). The concentration levels of IL-8 from stimulated A549 cells are shown in Figure 6. It is evident that when A549 cells are stimulated with the modified hexamer HEX17, IL-8 levels are significantly lower than when compared to Sp2CBMTD (aka SpOrig), or Hex6-stimulated cells.

[0141] Inflammatory mediator response using a Human Cytokine 12-plex Assay (Bio-Plex Pro™, Bio-Rad). Figure 7 demonstrates the analysis of 12 inflammatory mediators from culture medium after A549 cell stimulation by Sp2CBMTD (WT, aka SpOrig), HEX6 and HEX17 (variants) at specific time points (6h, 24h, 48h). Prior to analysis, samples were thawed and diluted 1:4 in PBS before using a human HS Cytokine-12 plex assay (R&D Systems). The data indicates that:

- HEX17 affects the levels of almost all the cytokines tested compared to SpOrig and HEX6. There is a significant reduction in observed concentration (pg/ml) with analytes IL-6, IL-8, GM-CSF and IFN-gamma at 48h when compared to SpOrig and HEX6.
- When compared to control at 48h, HEX17 appears to cause an increase in the level of all cytokines tested with the exception of IL-5, and VEGF (yet to be confirmed).
- HEX6 only showed reduced IL-6 stimulation compared to SpOrig at 48h.

EXAMPLE 2: *In vivo* PR8 mouse data.

[0142] The objective of the study was to assess the efficacy of Sp2CBMTD (SpOrig) and its variants, in a mouse model of lethal influenza infection. Each of the candidate proteins were also administered in the absence of an influenza infection to assess whether they alone, caused any morbidity or mortality.

Survival, clinical scores and weight loss.

[0143] The results show that none of CBM2 (HEX17), CBM3 (HEX6) or CBM4 (WT, SpOrig) caused any overt morbidity or mortality alone. Administration of a single 100µg dose of either CBM2 (HEX17), CBM3 (HEX6) and CBM4 (WT, SpOrig) one day prior to a lethal challenge with PR8 influenza virus elicited protection against PR8 infection, with greatest efficacy seen with HEX17 (100% survival), followed by SpOrig and then HEX6 (Figure 8). Clinical scores were also lower with HEX17 compared to SpOrig and HEX6 (Figure 9). Mice from single high dose treated groups that survived all lost weight at peak infection but soon recovered, in contrast to untreated, infected mice (Figure 10).

Anti-mCBM antibody analysis of lung homogenates and sera tissue from a PR8-challenged mouse study.

[0144] The objective of this study was to determine whether modified immunogenic epitopes of the modified

variants of Sp2CBMTD (SpOrig) demonstrated reduced antibody levels in mice in a PR8-challenged study (it should be noted that epitopes were modified based on human MHC-class II binding information). For this, survived mice from PR8-challenged study were culled at Day 21 with lung and sera harvested and tested for anti-mCBM antibodies - IgG, IgA, IgE and IgM against coated antigen SpOrig (1µg/well) in an ELISA format. The data shown in Figure 11 indicated that:

- The modified protein HEX17 did show a significant ($p<0.05$) reduction in mouse lung IgM levels compared to SPORIG. Due to only one surviving mouse for HEX6 treatment, only HEX17 and SPORIG data was statistically analysed.
- There is some indication of a slight downward trend of antibody levels in mice (lung IgA and IgM) that were treated with the modified CBMs compared to SpOrig.

Example 3: The Effect of Repeat Intranasal Dosing of mCBMs, Sp2CBMTD and HEX17, in the Mouse.

[0145] The objective of this study was to assess the clinical effect and immune response of repeat dosing of both Sp2CBMTD (SpOrig) and HEX17 in mice over time.

Experimental Procedures

[0146] Intranasal Dosing. On Days 1, 15 and 29, cohorts of mice (10F, 10M BALB/c mice per agent) were dosed with 20 µg of either sterile PBS, Sp2CBMTD or HEX17 via the intranasal route, under recoverable gaseous anaesthesia (isoflurane/oxygen mix), at a fixed volume of 40 µL.

[0147] Body Weights and Post-dose Observations. All mice were weighed twice weekly from Day -1 until the end of the study (Day 35). Post-dose observations were recorded every 15 min for the first 2 h after dose administration and then every 30 min for the next 6h.

[0148] Analysis of tissue samples. Mouse tissue (serum and bronchoalveolar lavage (BAL)) were tested for anti-CBM antibody responses (IgG, IgA and IgM) against either Sp2CBMTD or HEX17 as coating antigens (1 µg /well) in an ELISA format. Absorbance readings at OD_{450nm} (reference background OD_{620nm}) were measured for each sample after reaction of an HRP-conjugated detection antibody with its chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB), to the different antibody types.

Results:

Clinical scores.

[0149] Further studies into the effect of repeat intranasal dosing of Sp2CBMTD and Hex17 in mice reveals that neither molecule had any significant effect on bodyweight or food consumption. Further, at later doses, HEX17 appears to be better tolerated, with some (resolving) clinical signs (including piloerection, hunched posture, underactivity, partially closed eyes and irregular breathing) being noted for a limited period after *Sp*2CBMTD administration.

Anti-mCBM antibody analysis of serum and BAL tissue from mice.

[0150] The data shown in Figure 12 indicated that after 35 days, where mice were given 3 doses of 20 µg CBM every two weeks between Days 1 and 29, an adaptive immune response was induced to both CBMs. HEX17 (an example of a modified or 'de-immunized" CBM where epitopes are modified based on human MHC-class II binding information as described previously) demonstrated a significant ($p<0.05$) reduction of IgA in both BAL and serum tissues compared to Sp2CBMTD-treated mice. This was observed against both coated antigens. The difference in IgA response between the two candidates was also significant between male and female mice. The difference in IgG response was more evident in BAL samples than in sera. There was also a significant reduction of IgM levels from both BAL and serum samples when tested against Sp2CBMTD, but this was not significant in HEX17-coated plates.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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

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- GB2017052805W [0078]
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MODIFICERET PROTEIN

PATENTKRAV

1. Sialinsyrebindende molekyle, der omfatter følgende sekvens:

GAMVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDPKAPAFYNLFSVSSAT
KKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTVEKPTAELPKG
RARLYVNGGLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIRNLTVYNRALT
PEEVQKRSGGGSGVIEKEDVETNASNGQRVDLSSELDKLKKLENATVHMEFKPDPKAPAF
YNLFSVSSATKKDEYFTMAVYNNTATLEGRGSDGKQFYNNYNDAPLKVKPGQWNSVTFTV
EKPTAELPKGRARLYVNGGLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVWGSNLQIR
NLTVYNRALTPEEVQKRSGGGSLGVPDFESDWFDVSSNSLYTLSHGLQRSPRRVVVEFARS
SSPSTWNIVMPSYFNDGGHKGSGAQVEVGSLNIKLGTGAAVWGTGYFGGIDNSATTRFAT
GYYRVRAWI

2. Sialinsyrebindende molekyle ifølge krav 1 til anvendelse i terapi eller som et lægemiddel.

3. Sialinsyrebindende molekyle til anvendelse ifølge krav 2, hvor det sialinsyrebindende molekyle modulerer immunresponser hos humane eller dyreindivider.

4. Sialinsyrebindende molekyle til anvendelse ifølge krav 2, hvor det sialinsyrebindende molekyle anvendes som et adjuvans.

5. Farmaceutisk sammensætning, der omfatter det sialinsyrebindende molekyle ifølge krav 1, formuleret sammen med én eller flere hjælpestoffer, bærere, adjuvanser og/eller buffere.

6. Farmaceutisk sammensætning ifølge krav 5, hvor sammensætningen er formuleret til oral, mucosal eller parenteral administration.

7. Farmaceutisk sammensætning ifølge krav 6, hvor sammensætningen er formuleret til intranasal administration eller inhalation.

8. Fremgangsmåde til fremstilling af den farmaceutiske sammensætning ifølge krav 5 til 7, og som omfatter formulering af det sialinsyrebindende molekyle ifølge krav 1 sammen med ét/én eller flere hjælpestoffer, bærere, adjuvanser og/eller buffere.

DRAWINGS

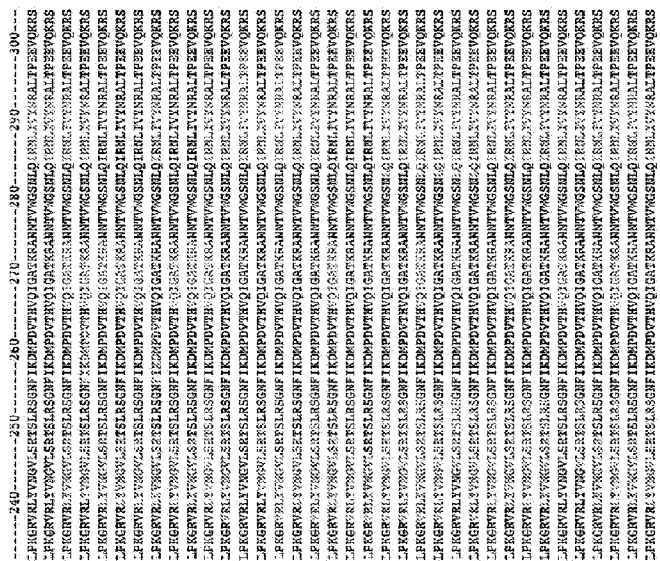


Figure 1A

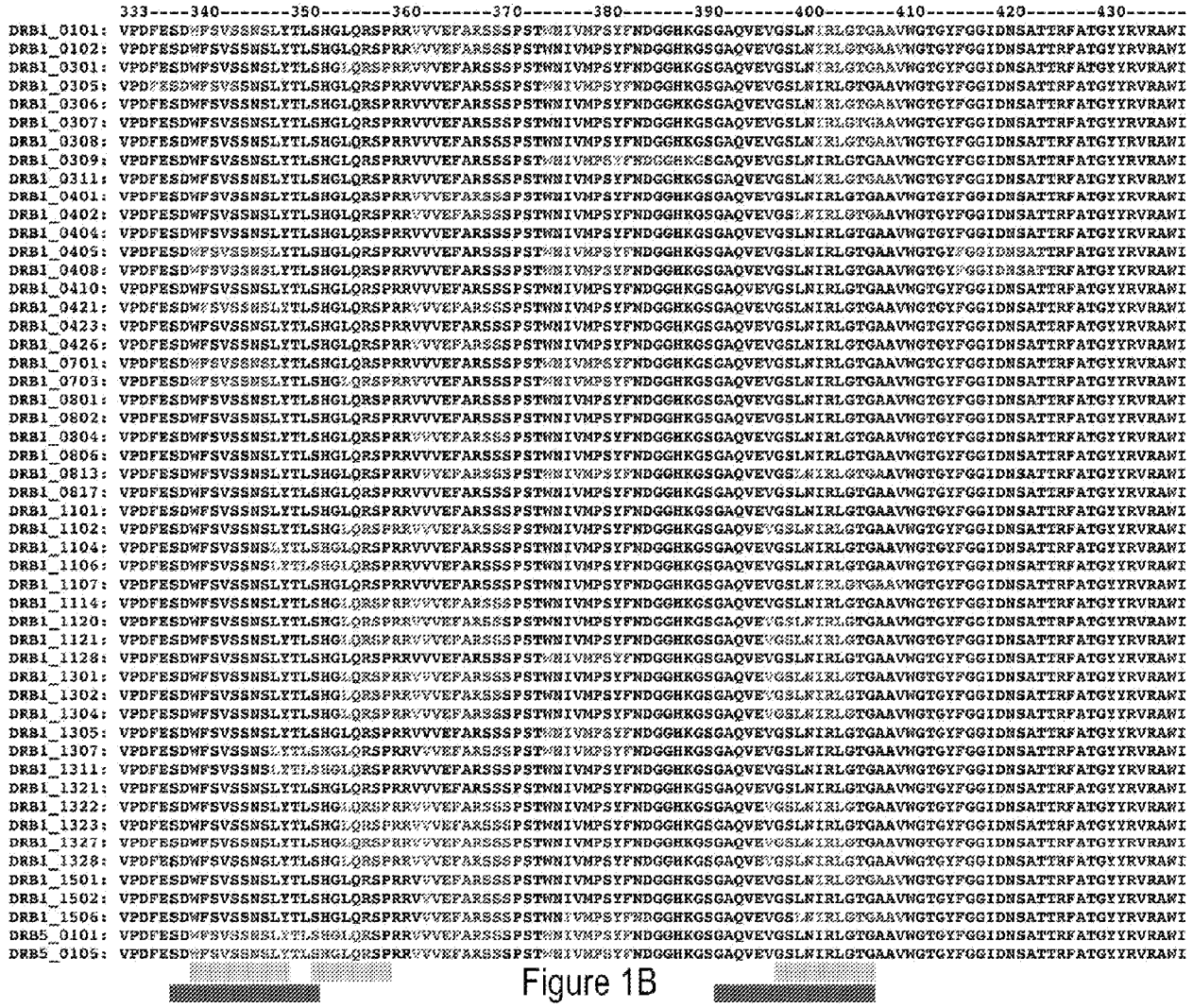

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Figure 1B

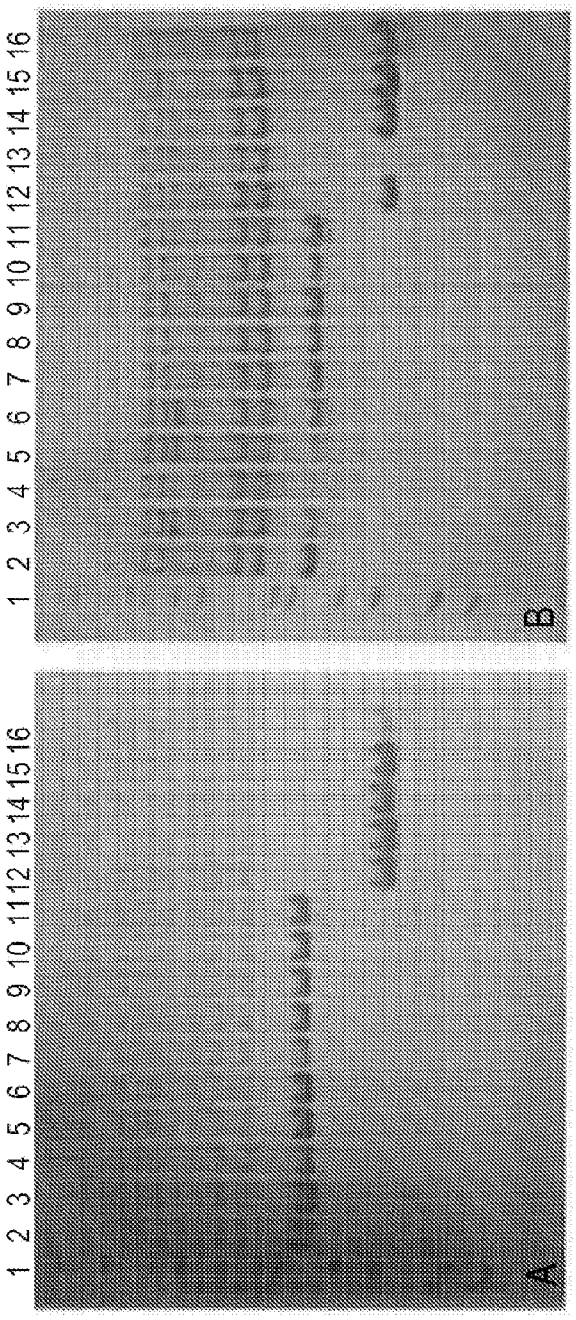


Figure 2

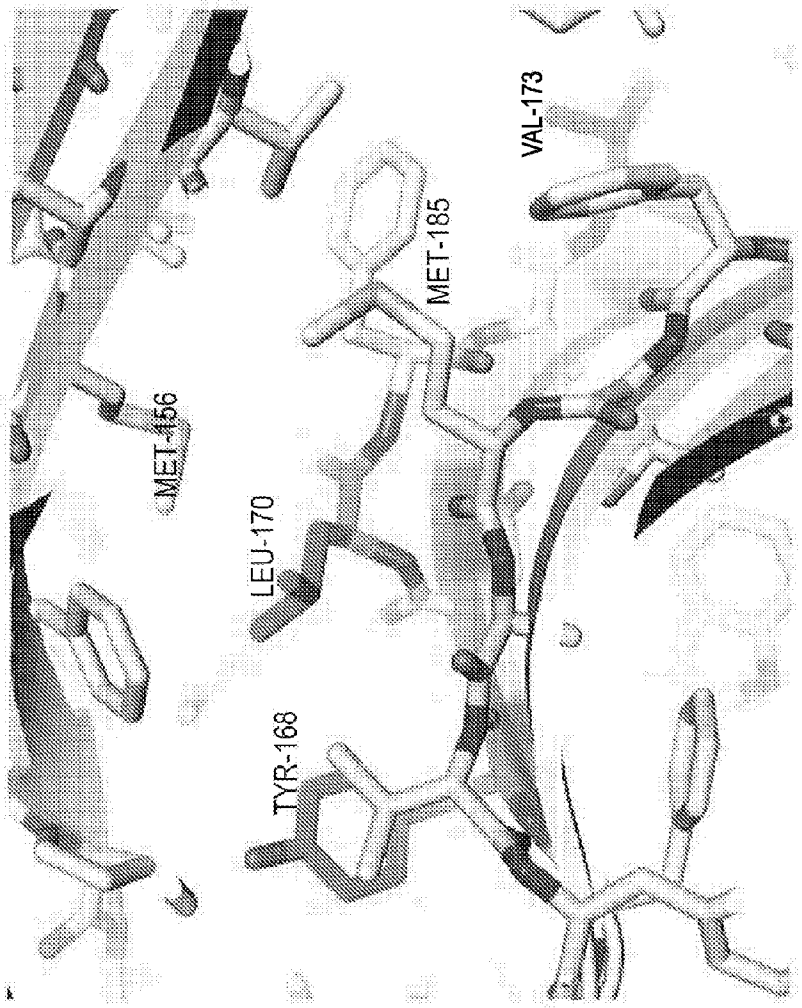


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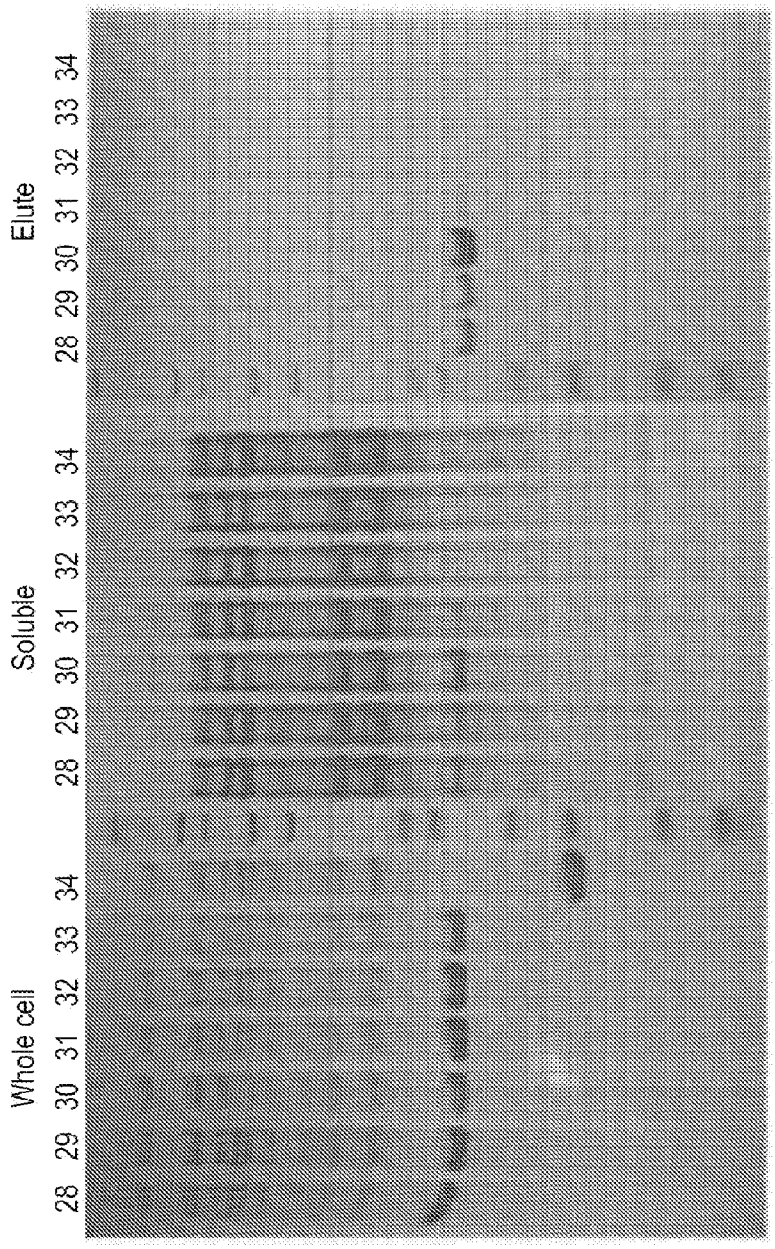

Whole cell
28 29 30 31 32 33 34
Soluble
28 29 30 31 32 33 34
Elute
28 29 30 31 32 33 34

Figure 4



Figure 5

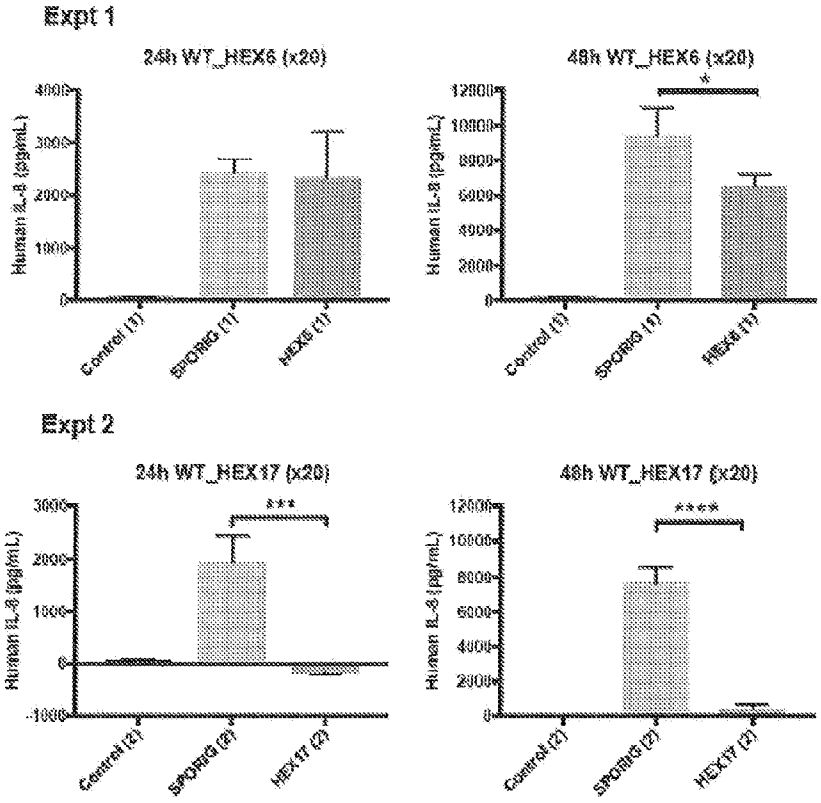

Human IL8 analysis of CBM-stimulated A549 cells
Expt 1
24h WT_HEX6 (x20)
48h WT_HEX6 (x20)
Human IL-8 (pg/mL)
Control (1)
SPORE6 (1)
HEX6 (1)
*
Expt 2
24h WT_HEX17 (x20)
48h WT_HEX17 (x20)
***
****
Control (2)
SPORE6 (2)
HEX17 (2)

Figure 6

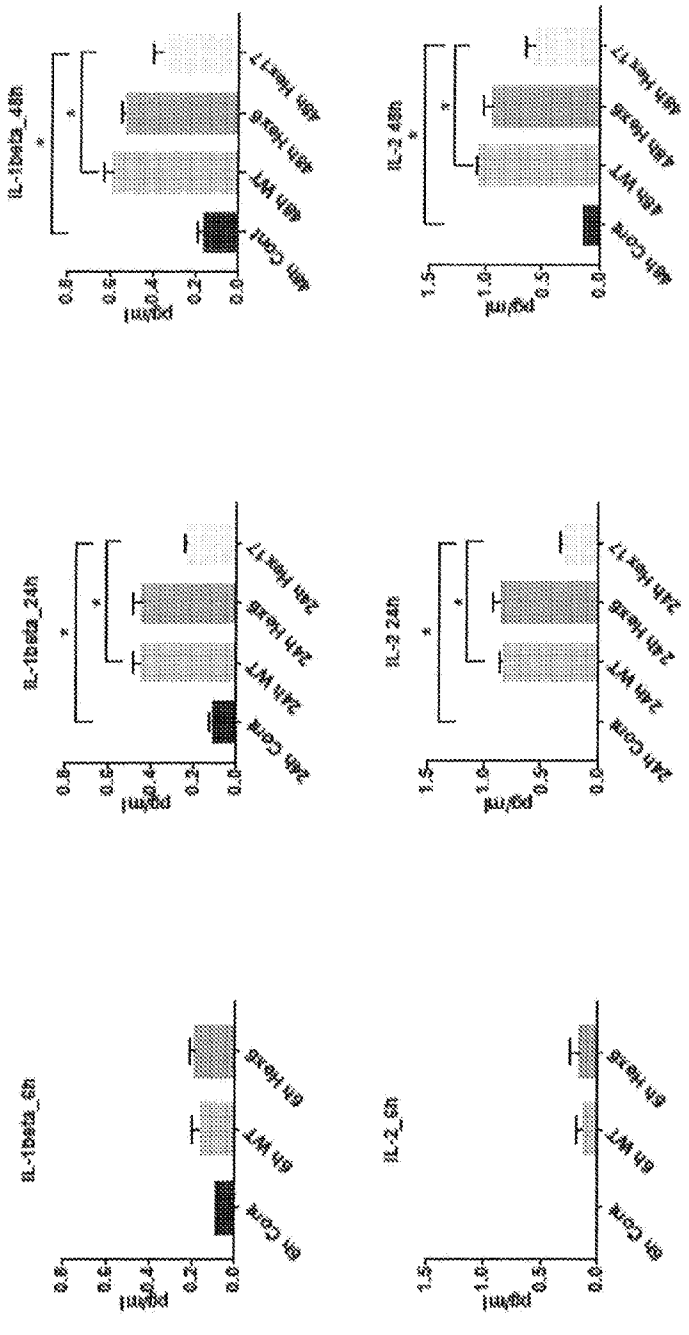

IL-1beta_6h
IL-2_6h
IL-1beta_24h
IL-2 24h
IL-1beta_48h
IL-2 48h
pg/ml
6dpi Cont
6dpi WT
6dpi Het5
6dpi Het7
24h Cont
24h WT
24h Het5
24h Het7
48h Cont
48h WT
48h Het5
48h Het7

Figure 7A

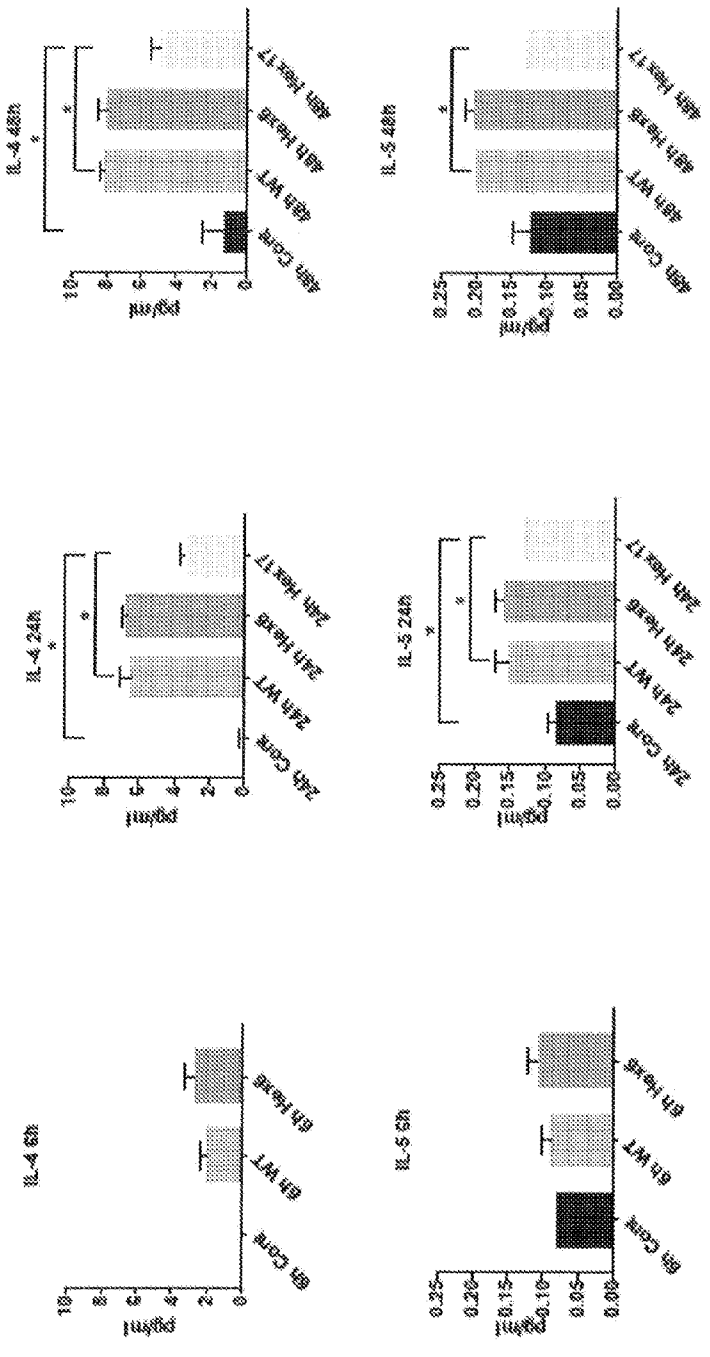

IL-4 48h
IL-4 24h
IL-4 6h
IL-5 48h
IL-5 24h
IL-5 6h
pg/ml
48h Cont
48h WT
48h Hes1fl
48h Hes1T
24h Cont
24h WT
24h Hes1fl
24h Hes1T
6h Cont
6h WT
6h Hes1fl

Figure 7B

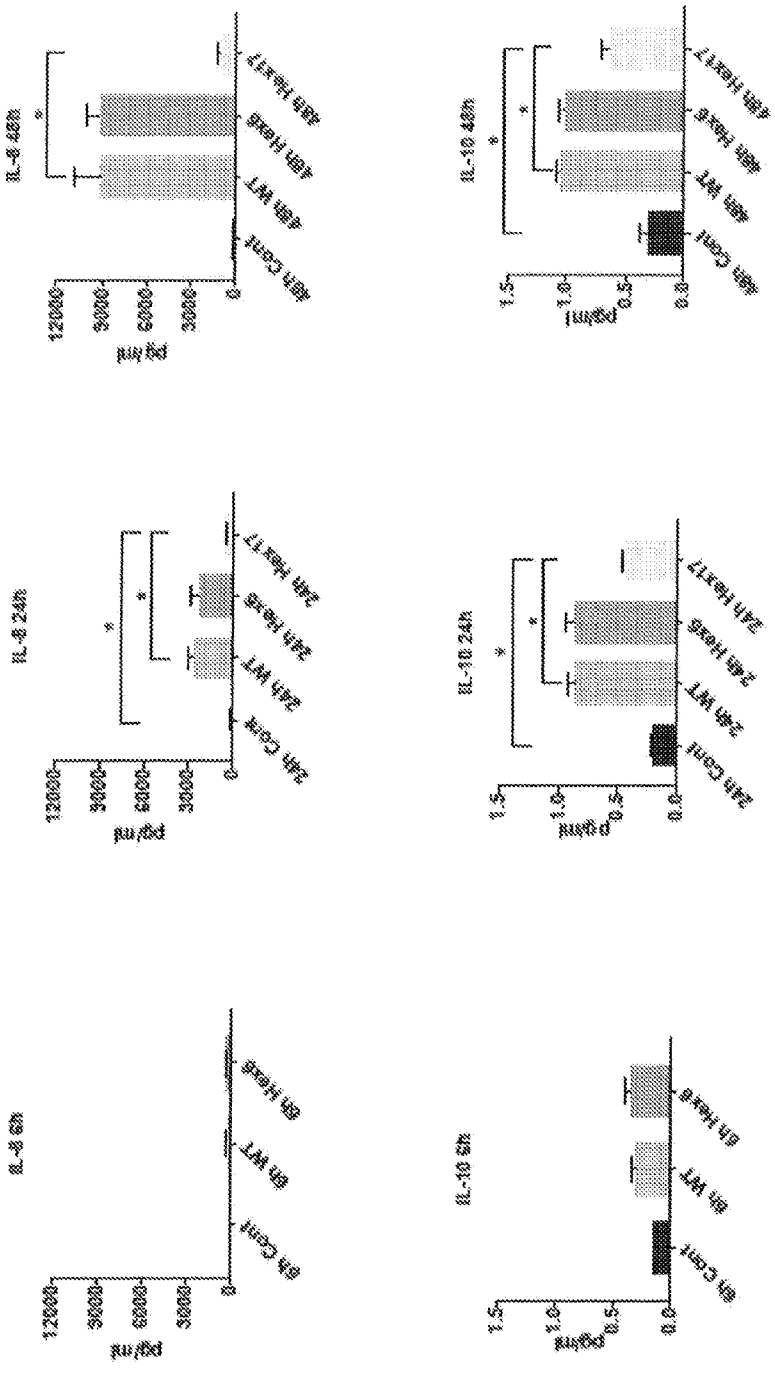

IL-6 48h
IL-6 24h
IL-6 6h
IL-10 48h
IL-10 24h
IL-10 6h
pg/ml
12000
9000
6000
3000
0
1.5
1.0
0.5
0.0
24h Cont
24h WT
24h Hets
24h Hets17
6h Cont
6h WT
6h Hets
*

Figure 7C

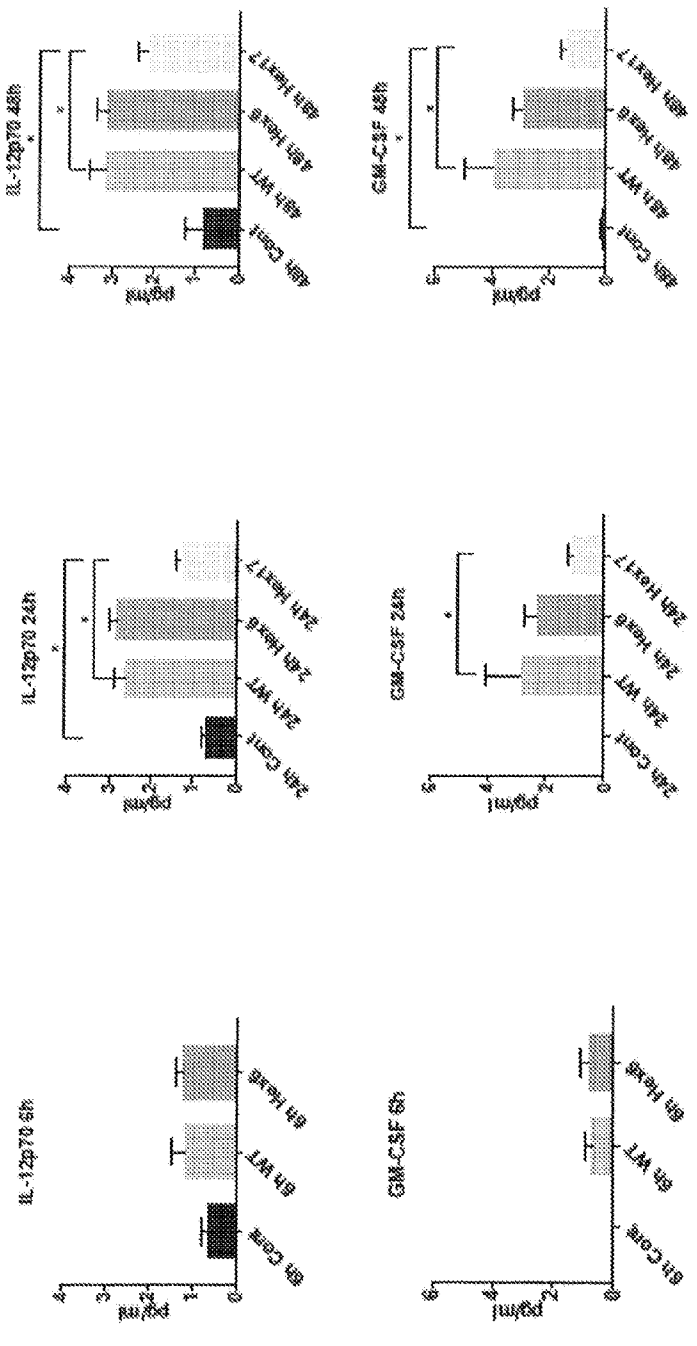

IL-12p70 6h
pg/ml
6h Cont
6h WT
6h Hexb
IL-12p70 24h
pg/ml
24h Cont
24h WT
24h Hexb
24h Hext7
IL-12p70 48h
pg/ml
48h Cont
48h WT
48h Hexb
48h Hext7
GM-CSF 6h
pg/ml
6h Cont
6h WT
6h Hexb
GM-CSF 24h
pg/ml
24h Cont
24h WT
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24h Hext7
GM-CSF 48h
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48h Cont
48h WT
48h Hexb
48h Hext7

Figure 7D

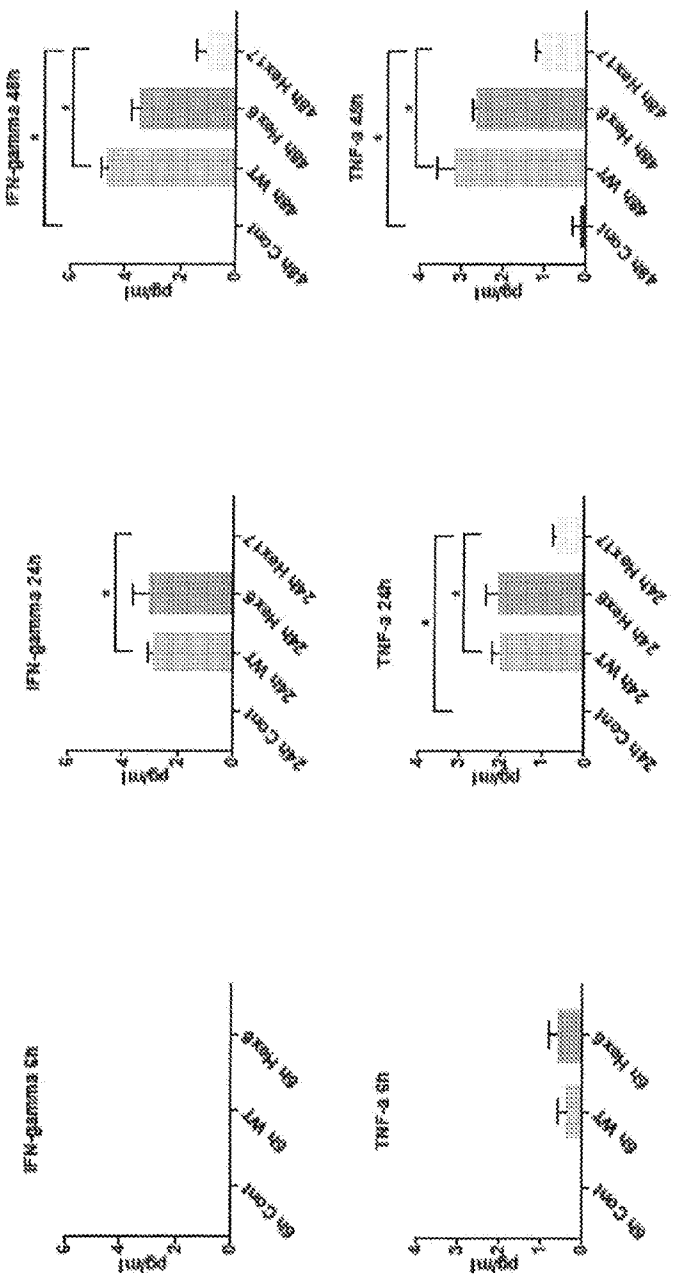

IFN-gamma 48h
TNF-a 48h
IFN-gamma 24h
TNF-a 24h
IFN-gamma 6h
TNF-a 6h
pg/ml
Adh Cont
Adh WT
Adh Hace
Adh Hace T
2dh Cont
2dh WT
2dh Hace
2dh Hace T
Gh Cont
Gh WT
Gh Hace

Figure 7E

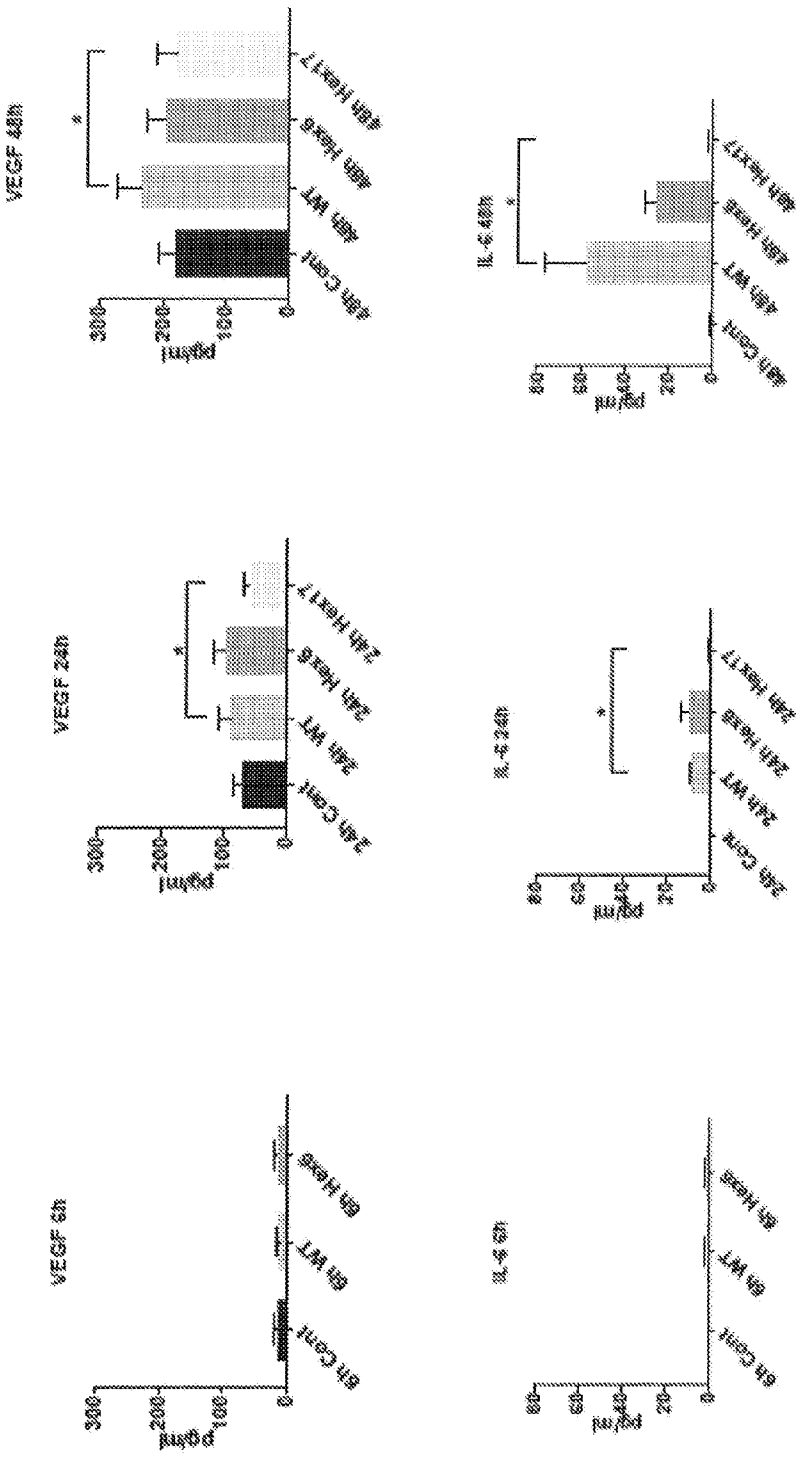

VEGF 48h
IL-6 48h
VEGF 24h
IL-6 24h
VEGF 6h
IL-6 6h
pg/ml
2dh Ctrl
2dh WT
2dh Hexb
2dh Hexb-T
6h Ctrl
6h WT
6h Hexb

Figure 7F

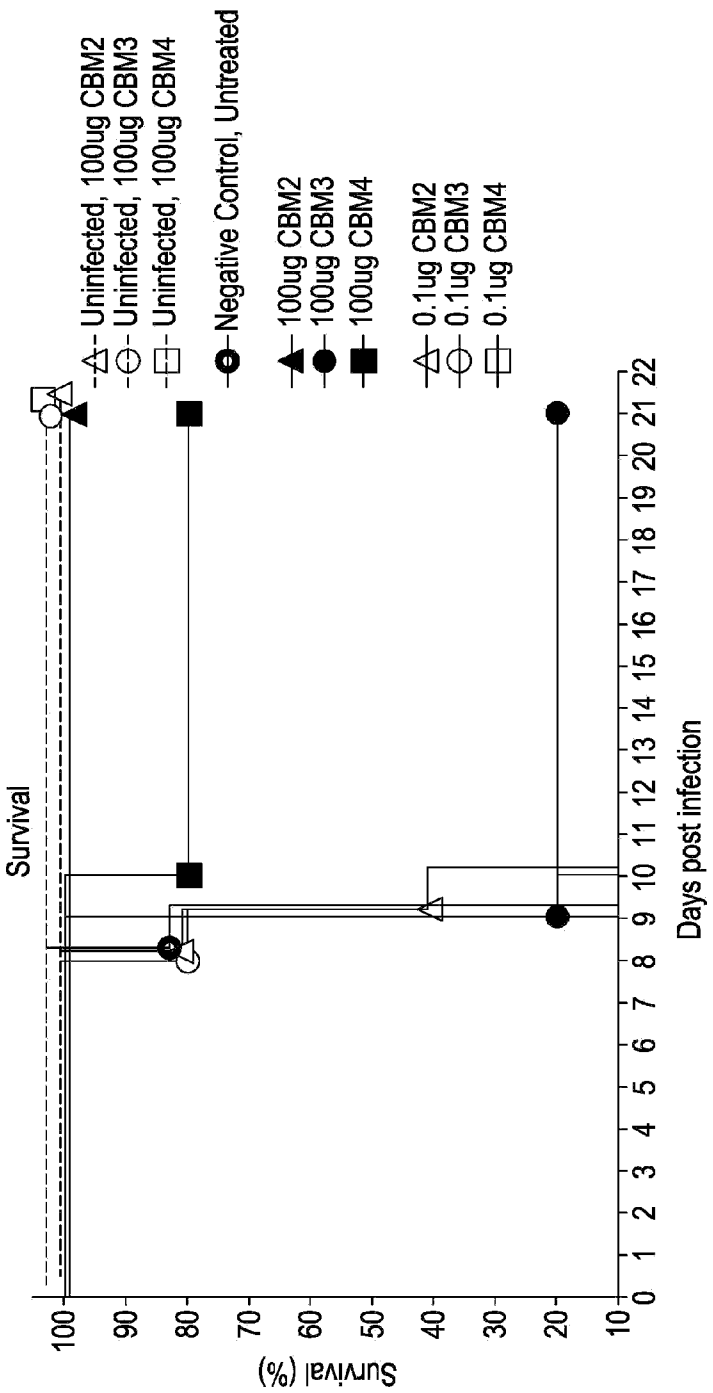

Survival
Survival (%)
100
90
80
70
60
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Days post infection
Uninfected, 100ug CBM2
Uninfected, 100ug CBM3
Uninfected, 100ug CBM4
Negative Control, Untreated
100ug CBM2
100ug CBM3
100ug CBM4
0.1ug CBM2
0.1ug CBM3
0.1ug CBM4

Figure 8

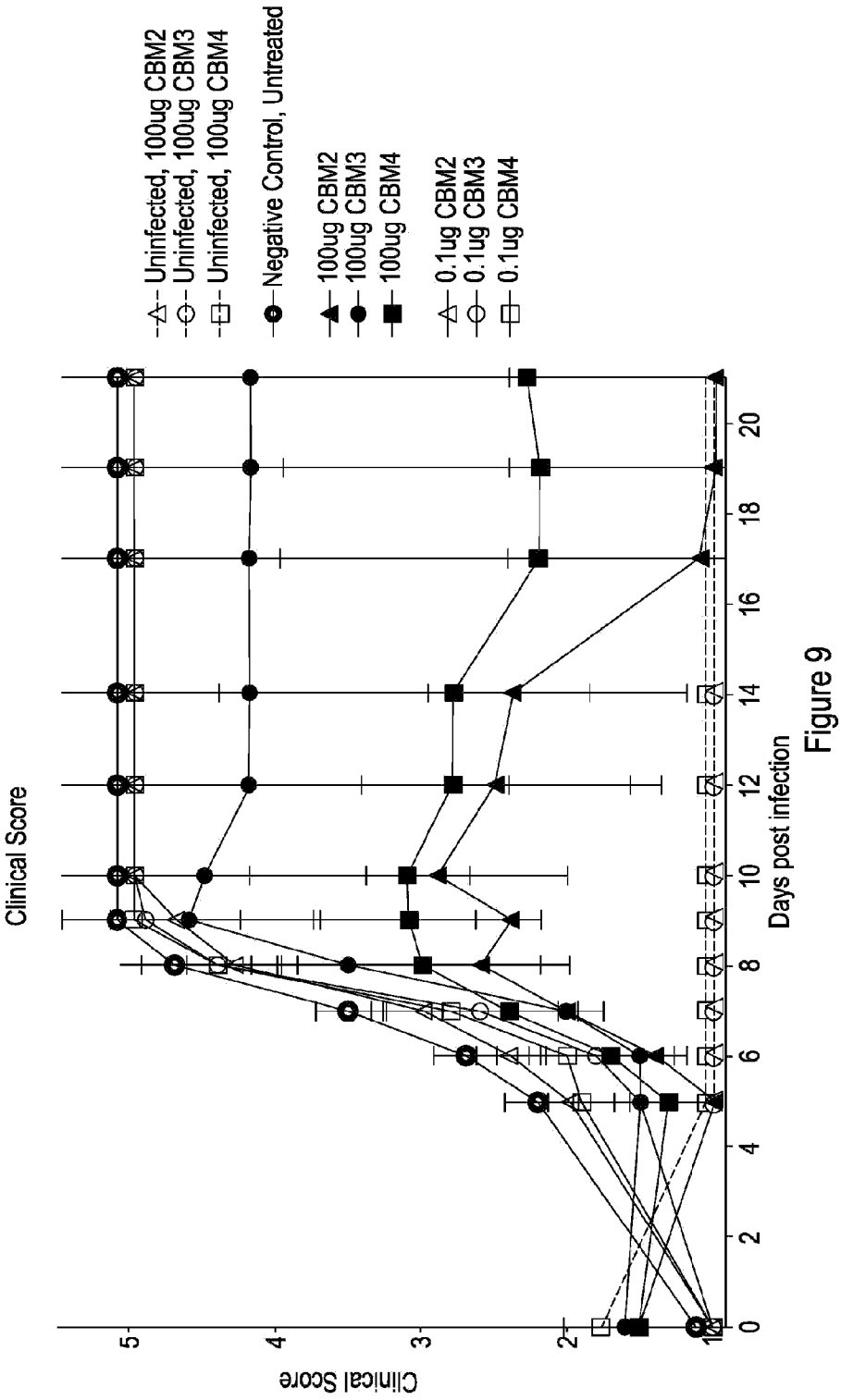

Clinical Score
Days post infection
Uninfected, 100ug CBM2
Uninfected, 100ug CBM3
Uninfected, 100ug CBM4
Negative Control, Untreated
100ug CBM2
100ug CBM3
100ug CBM4
0.1ug CBM2
0.1ug CBM3
0.1ug CBM4

Figure 9

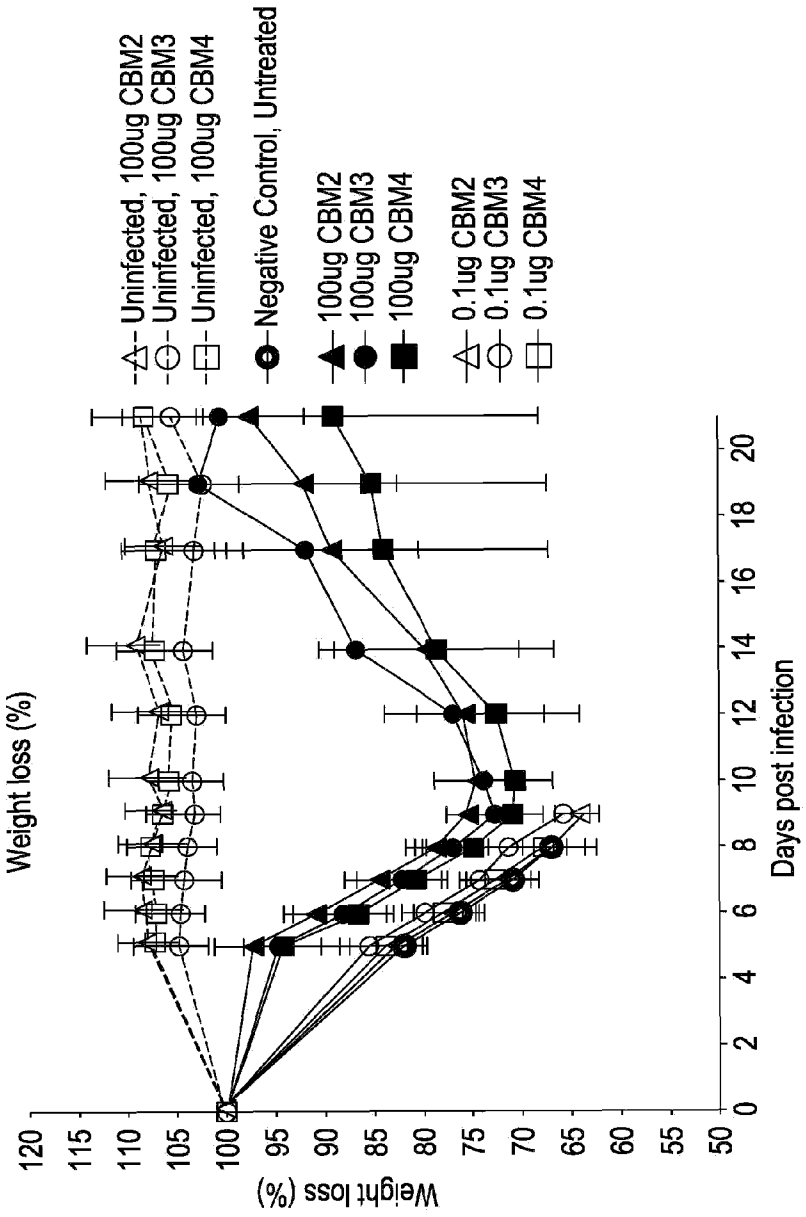

Uninfected, 100ug CBM2
Uninfected, 100ug CBM3
Uninfected, 100ug CBM4
Negative Control, Untreated
100ug CBM2
100ug CBM3
100ug CBM4
0.1ug CBM2
0.1ug CBM3
0.1ug CBM4
Weight loss (%)
Weight loss (%)
Days post infection
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
0
2
4
6
8
10
12
14
16
18
20

Figure 10

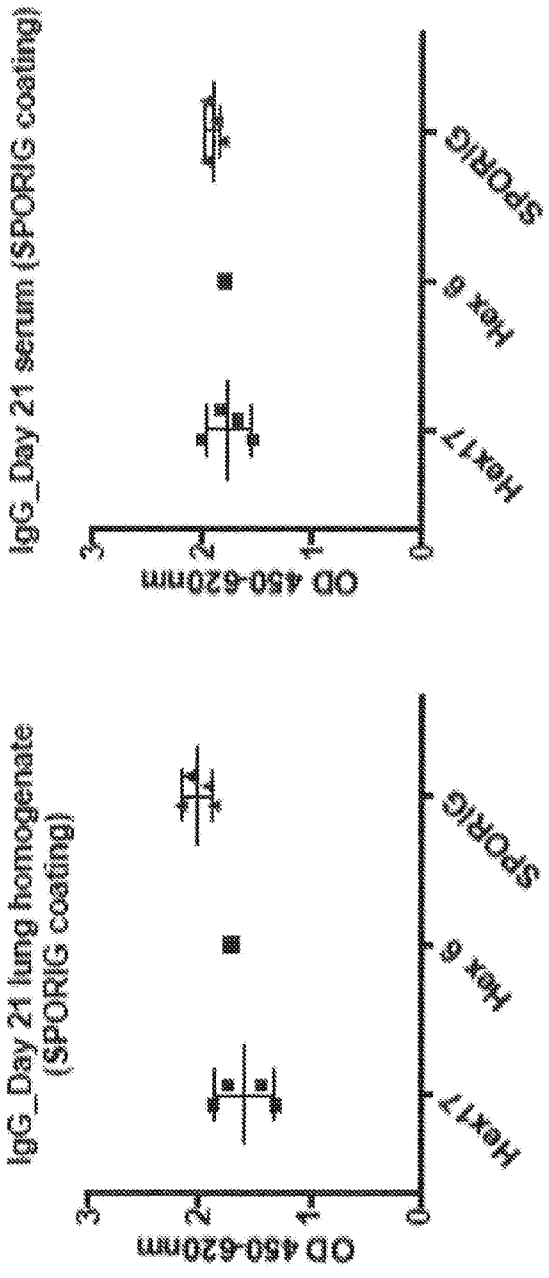

IgG_Day 21 serum (SPORIG coating)
OD 450-620nm
0
1
2
3
Hex17
Hex 6
SPORIG
IgG_Day 21 lung homogenate (SPORIG coating)
OD 450-620nm
0
1
2
3
Hex17
Hex 6
SPORIG

Figure 11A

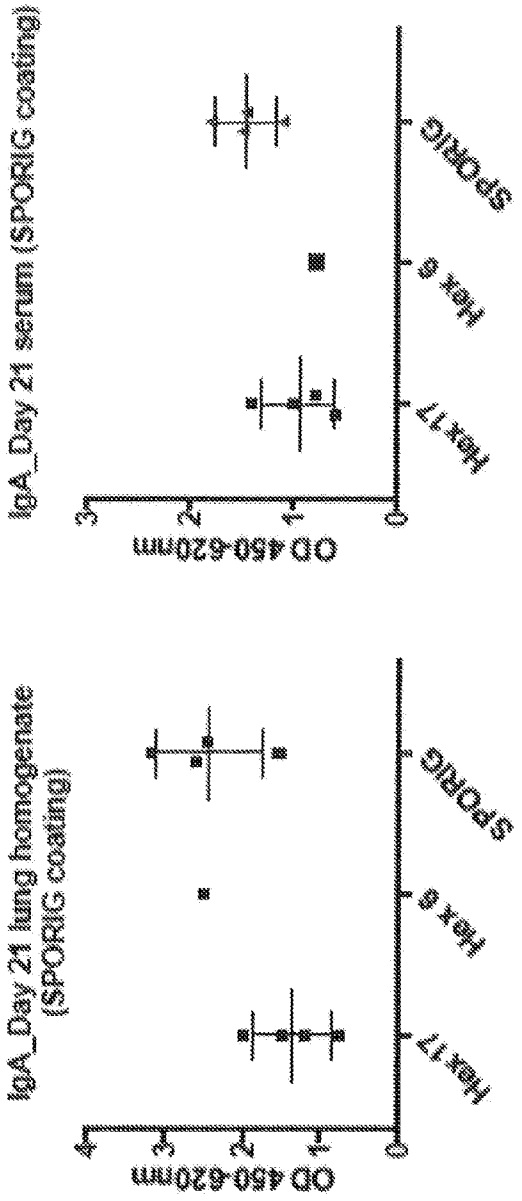

IgA_Day 21 serum (SPORIG coating)
OD 450-620nm
0
1
2
3
Hex 17
Hex 6
SPORIG
IgA_Day 21 lung homogenate (SPORIG coating)
OD 450-620nm
0
1
2
3
4
Hex 17
Hex 6
SPORIG

Figure 11B

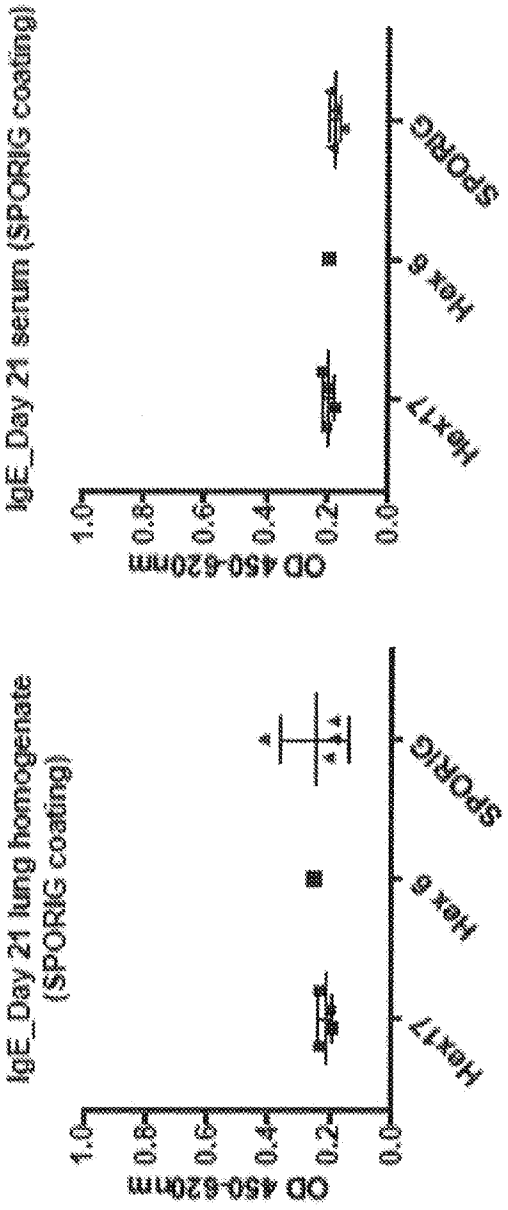

IgE_Day 21 serum (SPORIG coating)
OD 450-620nm
1.0
0.8
0.6
0.4
0.2
0.0
HeXT7
Hex 6
SPORIG
IgE_Day 21 lung homogenate (SPORIG coating)
OD 450-620nm
1.0
0.8
0.6
0.4
0.2
0.0
HeXT7
Hex 6
SPORIG

Figure 11C

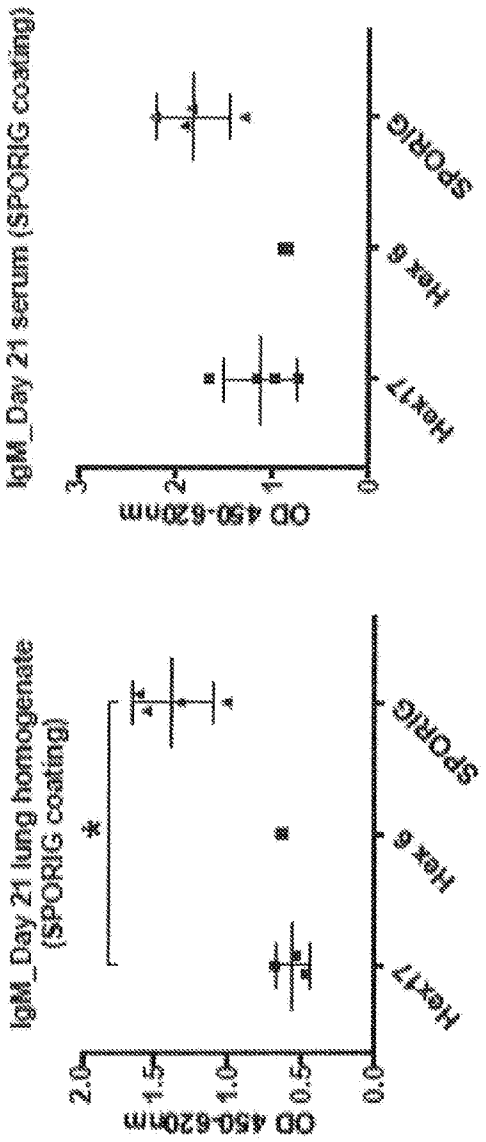

IgM_Day 21 lung homogenate (SPORIG coating)
OD 450-620nm
2.0
1.5
1.0
0.5
0.0
SPORIG
Hex 6
Hex17
IgM_Day 21 serum (SPORIG coating)
OD 450-620nm
3
2
1
0
SPORIG
Hex 6
Hex17

Figure 11D

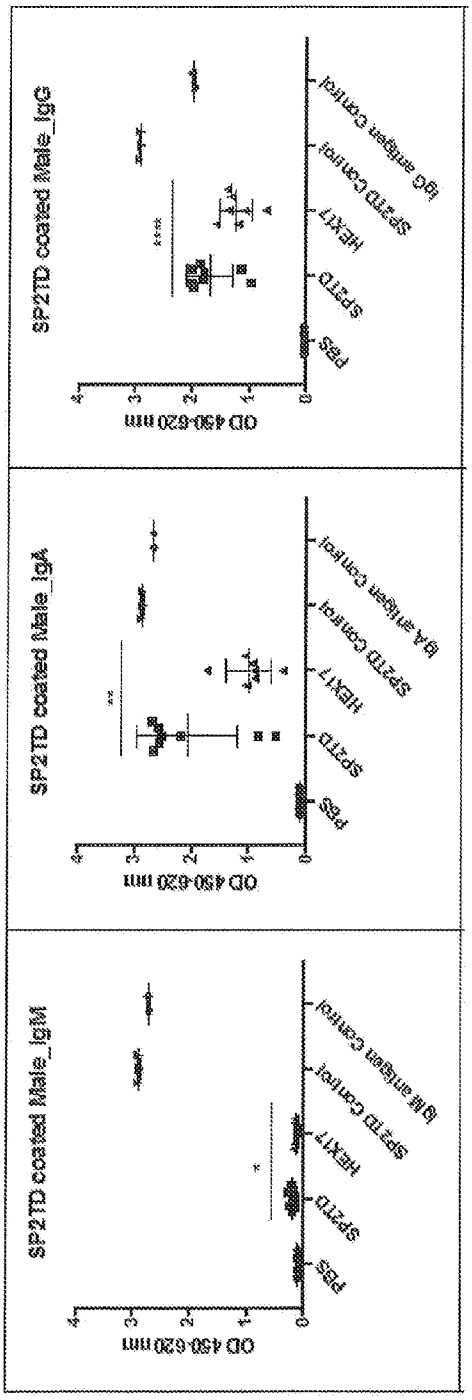

SP2TD coated Male_IgM
SP2TD coated Male_IgA
SP2TD coated Male_IgG
OD 450-620 nm
PBS
SP2TD
HEK17
SP2TD Control
IgM antigen Control
IgA antigen Control
IgG antigen Control

Figure 12(a)

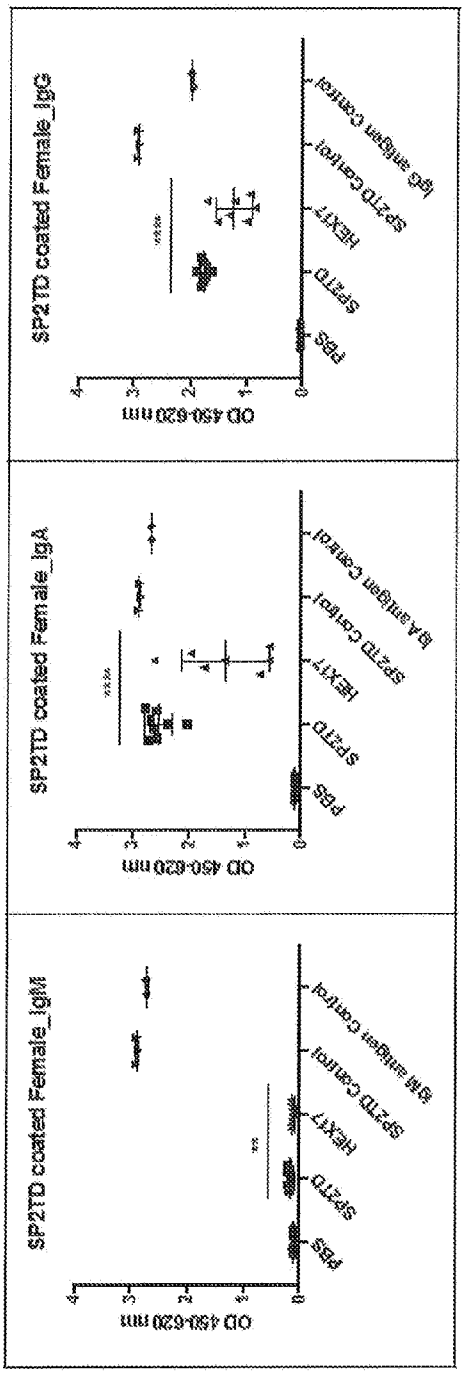


Figure 12a (continued)

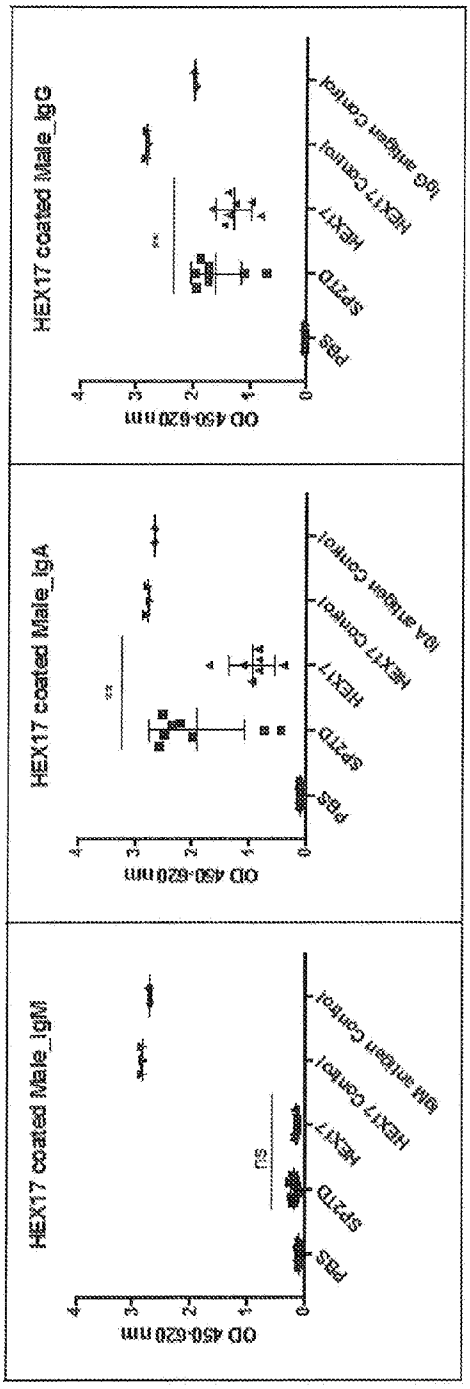


Figure 12a (continued)

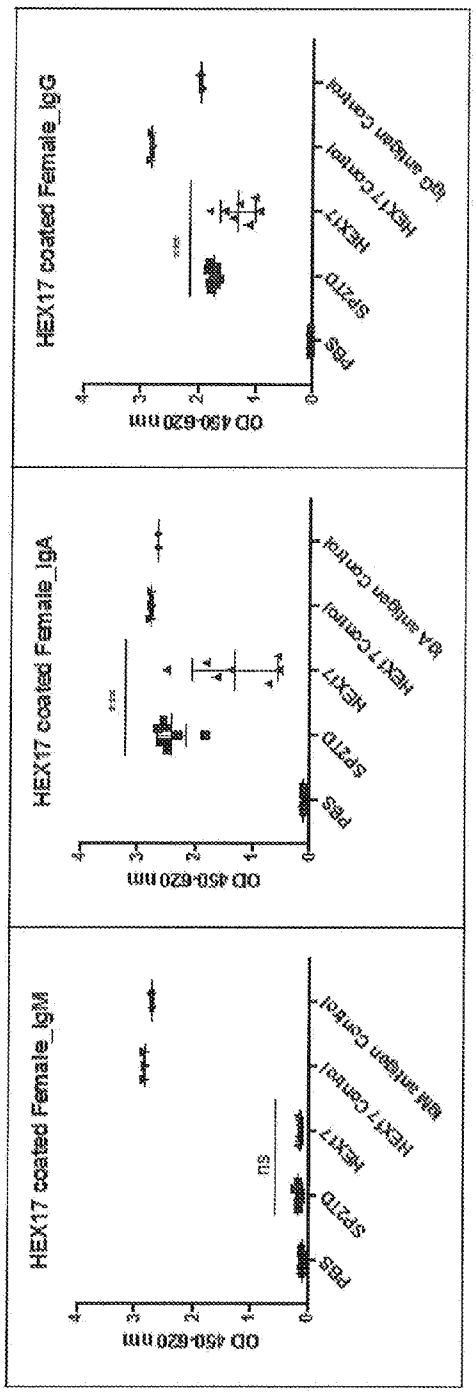


Figure 12a continued

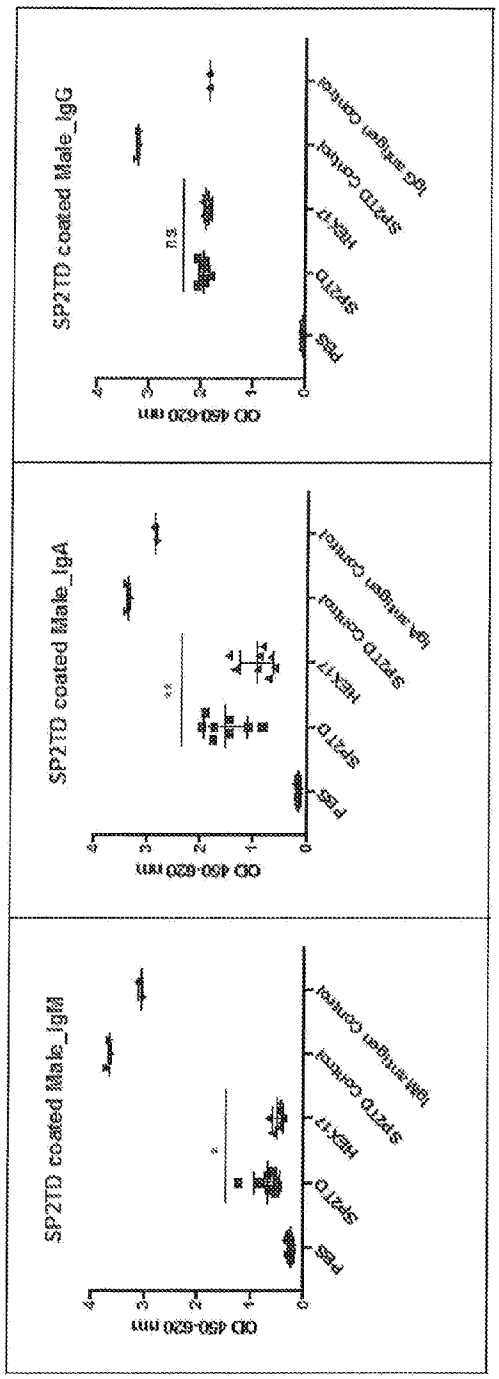

SP2TD coated Male_IgM
SP2TD coated Male_IgA
SP2TD coated Male_IgG
OD 450-620 nm
PBS
SP2TD
HEK17
SP2TD Control
IgM antigen Control
IgA antigen Control
IgG antigen Control
n.s

Figure 12(b)

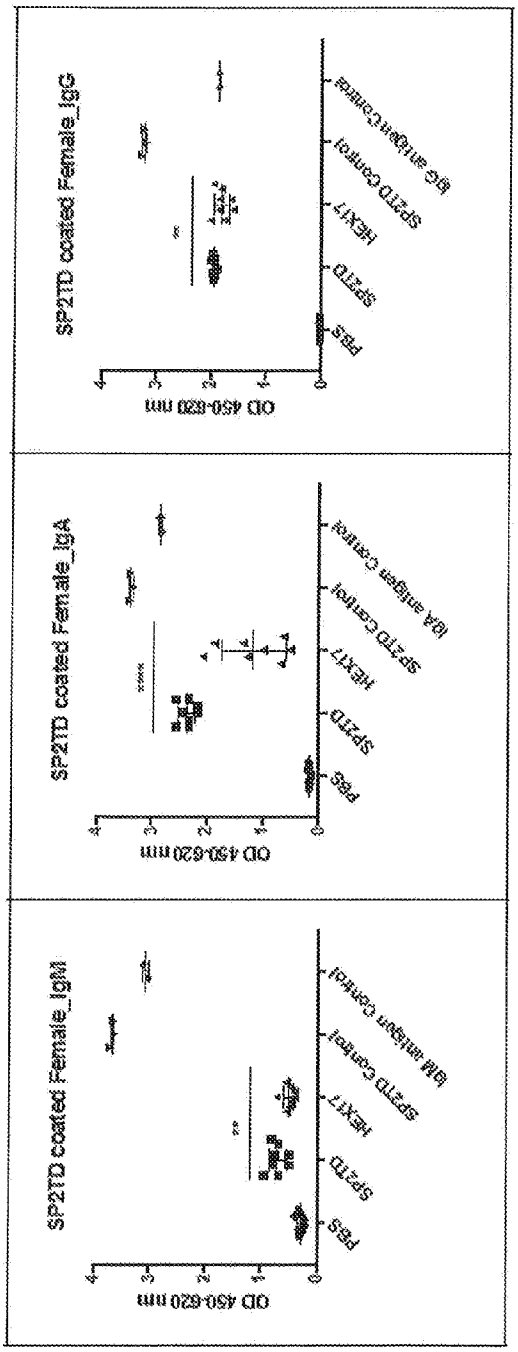


Figure 12(b) continued

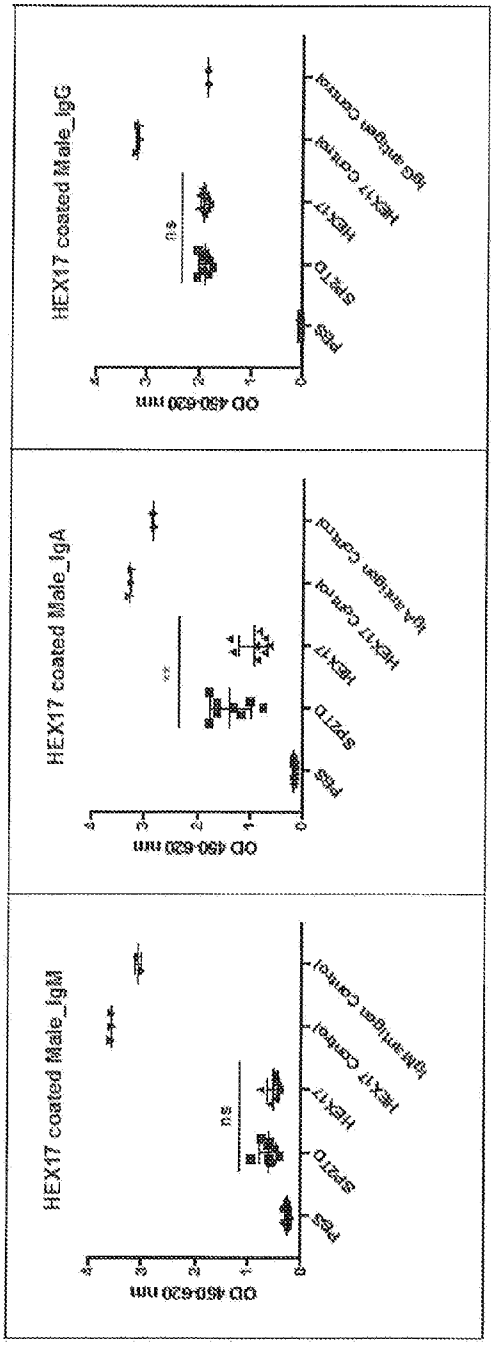


Figure 12(b) continued

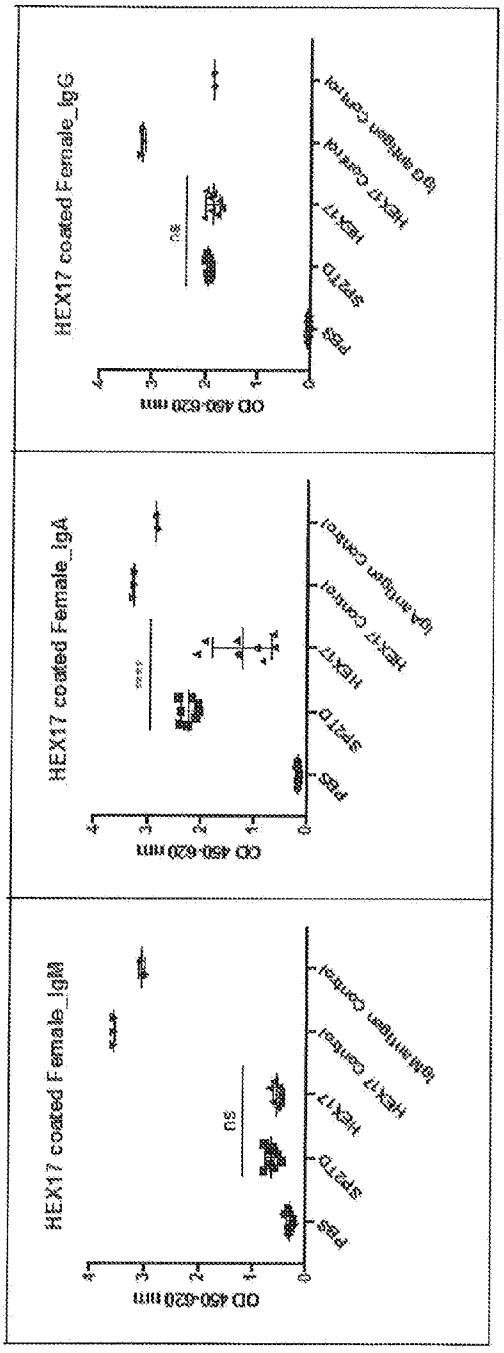


Figure 12(b) continued

SEKVENSLISTE

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

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