

BINDING MEMBERS

The present invention relates to specific binding members, such as antibodies and fragments thereof, that are capable of specifically binding a Lewis Y (Le^y) carbohydrate.

- 5 It also relates to the use of such binding members in medicine and to nucleic acids encoding such binding members.

Glycan structures are present on both protein and glycolipid backbones and can be massively over-expressed in cancer due to altered expression of glycosyltransferases.

- 10 Glycolipids consist of a lipid tail with a carbohydrate head and constitute about 5% of lipid molecules in the outer monolayer. There are three main series of glycolipids, globo, neolacto and ganglio lipids. Glycolipids are postulated to be very good targets due to their high surface density, mobility, and association with membrane microdomains; all of which contribute to strong cellular interactions. However, generating anti-glycolipid
- 15 antibodies is a challenging task as there is no T cell help and the monoclonal antibodies (mAbs) are usually of low affinity and of the IgM subclass (Durrant, Noble et al. 2012, Rabu, McIntosh et al. 2012). Although mAbs to glycans expressed on proteins overcome this problem, they present new challenges as the mAbs rarely see just the small glycan but usually recognise the glycan on the specific protein, giving a very restrictive expression.

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Only a limited number of antibodies recognising glycans have been described. Several anti-Lewis (Le) carbohydrate antigen mAbs have been generated to date but they often have cross reactivity with a range of glycans expressed on normal tissues. Lewis carbohydrate antigens are formed by the sequential addition of fucose onto oligosaccharide

25 precursor chains on glycoproteins or glycolipids through the action of a set of glycosyltransferases (Yuriev, Farrugia et al. 2005). They can be divided into 2 groups, type I (Le^a and Le^b) and type II (Le^x and Le^y). Le^a and Le^b antigens are regarded as blood group antigens whereas Le^x and Le^y are reported to be expressed only in certain epithelial cells (Scott, Geleick et al. 2000), viewed as tumour associated markers (Soejima and

30 Koda 2005) and represent attractive targets for cancer treatment, including antibody based immunotherapy (Scott, Geleick et al. 2000). Most normal tissues do not express Lewis Y, with the exception of epithelial cells in the gastrointestinal tract, but at very low densities (Schuster, Umana et al. 2005). However, Lewis Y (Fuca1-2Galb1-4(Fuca1-3)GlcNAc) is reported to be expressed at high densities on nearly 70% of human epithelial

35 carcinomas and thus is an attractive candidate target for cancer immunotherapy. The recognition of Lewis Y as a good therapeutic target has led to the production of a number of anti-Le^y antibodies. Some of these anti-Le^y mAbs cross-react with other Lewis antigens, for example the mAb 692/29 which recognises Le^{b/y}, have demonstrated anti-tumour responses against colonic tumours in xenograft models (Noble, Spendlove et al.

40 2013).

Several other mAbs that bind Le^y are known in the art, but they cross react with other related Lewis antigens. For example, EP-B-0285059 discloses an antibody, BR-55, which reacts with both Lewis^y and B-7-2. B-7-2 has also been shown to be associated with tumour cells
5 (EP-B-0285059). US5869045 discloses an antibody, BR-96, which binds to both Lewis^y and Lewis^x haptens. Antibodies which bind to both Lewis^y and Lewis^b antigens are known. Studies have demonstrated that C14 monoclonal antibody recognises and binds to both Lewis^y and Lewis^b (extended and non-extended forms) antigens (Durrant et al., Hybridoma, 12, 647-660 (1996)). WO02/092126 discloses mAbs that bind to both Lewis^y and Lewis^b
10 haptens.

A number of clinical trials utilising murine or chimeric anti-Le^y antibodies have been conducted (Scott, Geleick et al. 2000). Unfortunately, often, anti-Le^y mAbs cross-react with Lewis X and H type II structures that are more commonly expressed on normal tissues
15 (Furukawa, Welt et al. 1990, Yin, Finstad et al. 1996). A range of Le^y antibodies has also been identified but a consistent problem with these antibodies has been the degree of cross reactivity with Le^x, and H type 2 structures causing red blood cell agglutination and gastrointestinal toxicity (Kitamura, Stockert et al. 1994, Pai, Wittes et al. 1996, Tolcher, Sugarman et al. 1999). Thus, fine specificity of the anti-Le^y antibodies is important for
20 successful clinical treatment.

In a first aspect, the present invention provides an isolated specific binding member capable of binding specifically to Fuca1-2Galb1-4(Fuca1-3)GlcNAc (Le^y).

25 The inventors have provided specific binding members which demonstrate potent *in vivo* anti-tumour activity. The specific binding members of the invention display potent immune-mediated cytotoxic activity against human colon cancer cells *in vitro* via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). They also have the ability to directly induce cell death without the need for
30 immune effector cells. By immunising with tumour glycolipid rather than glycoproteins or whole cells, the inventors have produced ultra-specific anti-Le^y mAbs, which do not cross react with any other glycan. Examples of these mAbs are an IgG3k murine mAb known herein as "FG27.10" and an IgG1k murine mAb known herein as "FG27.18", a hIgG1 chimeric (known as "CH27") and hIgG1 humanised (known as "Hu27").
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In contrast to previous studies with anti-Le^y mAbs that cross react with other glycans, the specific binding members of the present invention have a very restrictive normal distribution and only bound to subpopulations of cells within normal stomach, lung, tonsil, pancreas and duodenum. Further, they failed to stain colon, jejunum, breast, kidney or the ileum. They bind strongly to a
40 wide range of tumours including 80% of gastric, 100% of colorectal, 95% of ovarian and 85% of

breast tumours. Specific binding members of the invention, such as FG27.10/18, have monospecificity for just Le^y. The inventors have shown that specific binding members in accordance with failed to bind to the cell line Colo21. By contrast, a Le^{y/x} mAb bound strongly and a Le^{y/b} mAb bound moderately. This shows that Colo201 expresses Le^x and Le^b, but not Le^y and

5 confirms the specificity of the specific binding members of the present invention. This specificity is reflected in the similar but distinct sequence of the FG27 variable heavy and light chain regions (Figure 13a,b). FG27 shows 16-68% differences in the amino acid sequences CDRs within the heavy chain, 4.3-16% differences in amino acid differences in the CDR sequence in the light chain, 8.6-49.5% differences in amino acid sequence in the variable region of the heavy chain, 2-24%

10 differences in amino acid sequence in the variable region of the light chain from the other cross reactive Le^y mAbs (see Table 4 herein). The most variation is within CDRH2 with 3-5 of the 8 amino acids varying between the different mAbs.

Specific binding members of the present invention may be attached to a lipid or protein

15 backbone. The protein backbone may have a molecular weight of about 50-150kDa, as determined by SDS-PAGE.

The specific binding member of the present invention preferably comprise one or more binding domains selected from the binding domains having the amino acid sequence

20 substantially as set out as residues 27 to 38 (CDRH1), 54 to 65 (CDRH2) or 105 to 116 (CDRH3) of Figure 1a or 1b. The specific binding member may comprise a binding domain which comprises an amino acid sequence substantially as set out as residues 105 to 116 (CDRH3) of the amino acid sequence of Figure 1a or 1b. Such a specific binding member may additionally comprise one or both, preferably both, of the binding

25 domains having an amino acid sequence substantially as set out as residues 27 to 38 (CDRH1) and residues 56 to 65 (CDRH2) of the amino acid sequence shown in Figure 1a and 1b.

The binding member may comprise an amino acid sequence substantially as set out as 1-

30 127 (VH) of Figure 1a or 1b.

The specific binding member may comprise one or more binding domains selected from binding domains having the amino acid sequence of residues 27 to 38 (CDRL1), 56 to 65 (CDRL2) or 105 to 113 (CDRL3) of Figure 1c. The binding member may comprise a binding

35 domain having an amino acid sequence substantially as set out as residues 105 to 113 (CDRL3) of the amino acid sequence of Figure 1c. Such a specific binding member may additionally comprise one or both, preferably both, of the binding domains having an amino acid sequence substantially as set out as residues 27 to 38 (CDRL1) and residues 56 to 65 (CDRL2) of the amino acid sequence shown in Figure 1c.

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Specific binding members which comprise a plurality of binding domains of the same or different sequence, or combinations thereof, are included within the present invention. The or each binding domain may be carried by a human antibody framework. For example, one or more binding domains may be substituted for the complementary determining regions (CDRs) of a whole human antibody or of the variable region thereof.

One isolated specific binding member of the invention comprises the amino acid sequence substantially as set out as residues 1 to 124 (VL) of the amino acid sequence shown in Figure 1c.

The isolated specific binding member of the invention may comprise one or more, preferably all, of the binding domains having an amino acid sequence substantially as set out as residues 27 to 38 (CDRH1), 54 to 65 (CDRH2) or 105 to 116 (CDRH3) of Figure 1a or 1b in combination with one or more, preferably all, of the binding domains having an amino acid sequence substantially as set out as residues 27 to 38 (CDRL1), 56 to 65 (CDRL2) or 105 to 113 (CDRL3) of Figure 1c.

The binding member may comprise an amino acid sequence substantially as set out as residues 1 to 127 (VH) of the amino acid sequence of Figure 1a or 1b, and an amino acid sequence substantially as set out as residues 1 to 124 (VL) of the amino acid sequence of Figure 1c.

Once a single, archetypal mAb, for example an FG27 mAb, has been isolated that has the desired properties described herein, it is straightforward to generate other mAbs with similar properties, by using art-known methods. For example, the method of Jespers *et al.*, 1994 (Jespers, Roberts et al. 1994), may be used to guide the selection of mAbs having the same epitope and therefore similar properties to the archetypal mAb. Using phage display, first the heavy chain of the archetypal antibody is paired with a repertoire of (preferably human) light chains to select a glycan-binding mAb, and then the new light chain is paired with a repertoire of (preferably human) heavy chains to select a (preferably human) glycan-binding mAb having the same epitope as the archetypal mAb.

The specific binding member may be an antibody or an antibody fragment, Fab, (Fab')₂, scFv, Fv, dAb, Fd or a diabody. The antibody may be a polyclonal antibody. The antibody may be a monoclonal antibody (mAb). Antibodies of the invention may be humanised, chimeric or veneered antibodies, or may be non-human antibodies of any species.

Murine or chimeric antibodies carry an increased risk of adverse anti-murine antibody (HAMA) reactions in patients (Schroff et al. 1985; Azinovic et al. 2006; Miotti et al. 1999; D'Arcy and Mannik 2001). Accordingly, most approved therapeutic mAbs are either

humanised or fully human IgG antibodies

The specific binding member of the invention may comprise a heavy chain having an amino acid sequence substantially as set out in Figure 2a and a light chain having an amino acid sequence substantially as set out in Figure 2b.

The specific binding member of the invention may comprise a heavy chain having an amino acid sequence substantially as set out in Figure 3a and a light chain having an amino acid sequence substantially as set out in Figure 3b.

The specific binding member of the invention may comprise a heavy chain having an amino acid sequence substantially as set out in Figure 33 and/or a light chain having an amino acid sequence substantially as set out in Figure 33.

The invention further provides a binding member which competes for binding to Le^y containing glycans with an antibody comprising a VH chain having the amino acid sequence of residues 1 to 127 of Figure 1a or 1b and a VL chain having the amino acid sequence of residues 1 to 124 of Figure 1c.

Specific binding members that are capable of binding specifically to Le^y and are at least 90%, at least 95% or at least 99% identical in the VH and/or VL domain to the VH or VL domains of Figure 1, 2 or 3 are included in the invention. Specific binding members that are capable of binding specifically to Le^y and are at least 90%, at least 95% or at least 99% identical to the heavy chain and/or light chain of Figure 33 are included in the invention. Preferably such antibodies differ from the sequences of Figure 1, 2 or 3 by a small number of functionally inconsequential amino acid substitutions (e.g., conservative substitutions), deletions, or insertions.

Specific binding members of the invention may carry a detectable or functional label.

In further aspects, the invention provides an isolated nucleic acid encoding a specific binding member of the invention, and methods of preparing specific binding members of the invention which comprise expressing said nucleic acids under conditions to bring about expression of said binding member, and recovering the binding member. Isolated nucleic acids encoding specific binding members that are capable of binding specifically to Le^y and are at least 90%, at least 95% or at least 99% identical to the sequences provided herein are included in the invention.

Specific binding members of the invention may be used in a method of treatment or diagnosis of the human or animal body, such as a method of treatment of a tumour in a

patient (preferably human) which comprises administering to said patient an effective amount of a specific binding member of the invention. The invention also provides a specific binding member of the present invention for use in medicine, preferably for use in treating a tumour, as well as the use of a specific binding member of the present invention in the manufacture of a medicament for the diagnosis or treatment of a tumour. The tumour may be a gastric, colorectal, pancreatic, lung, ovarian or breast tumour.

Disclosed herein is the antigen to which the specific binding members of the present invention bind. A Le^y which is capable of being bound, preferably specifically, by a specific binding member of the present invention may be provided. The Le^y may be provided in isolated form, and may be used in a screen to develop further specific binding members therefor. For example, a library of compounds may be screened for members of the library which bind specifically to the Le^y. The Le^y may be on a lipid or protein backbone. When on a protein backbone, it may have a molecular weight of about 50-150kDa, as determined by SDS-PAGE.

In a further aspect the invention provides an isolated specific binding member capable of binding Le^y containing glycans, preferably of the first aspect of the invention, for use in the diagnosis or prognosis of gastric, colorectal, pancreatic, lung, ovarian and breast tumours.

The invention further provides a method for diagnosis of cancer comprising using a specific binding member of the invention to detect Le^y containing glycans in a sample from an individual. In the diagnostic method, the pattern of glycans detected by the binding member may be used to stratify therapy options for the individual.

These and other aspects of the invention are described in further detail below.

As used herein, a "specific binding member" is a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its surface, which may be a protrusion or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus, the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, 5 receptor-ligand, enzyme-substrate. The present invention is generally concerned with antigen-antibody type reactions, although it also concerns small molecules which bind to the antigen defined herein.

As used herein, "treatment" includes any regime that can benefit a human or non-human

animal, preferably mammal. The treatment may be in respect of an existing condition or may be prophylactic (preventative treatment).

As used herein, a "tumour" is an abnormal growth of tissue. It may be localised (benign) or invade nearby tissues (malignant) or distant tissues (metastatic). Tumours include neoplastic growths which cause cancer and include oesophageal, colorectal, gastric, breast, ovarian and endometrial tumours, as well as cancerous tissues or cell lines including, but not limited to, leukaemic cells. As used herein, "tumour" also includes within its scope endometriosis.

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The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds an antigen, whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies of the invention are the immunoglobulin isotypes (*e.g.*, IgG, IgE, IgM, IgD and IgA) and their isotypic subclasses; fragments which comprises an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies. Antibodies may be polyclonal or monoclonal. A monoclonal antibody may be referred to as a "mAb".

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It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the CDRs, of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

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As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, humanised antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023. A humanised antibody may be a modified antibody having the variable regions of a non-human, *e.g.*, murine, antibody and the constant region of a human antibody. Methods for making

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humanised antibodies are described in, for example, US Patent No. 5225539.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, Gussow et al. 1989) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments; (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird, Hardman et al. 1988, Huston, Levinson et al. 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and; (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; (Holliger, Prospero et al. 1993)).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (e.g., by a peptide linker) but unable to associated with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger and Winter 1993), e.g., prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in (Traunecker, Lanzavecchia et al. 1991).

Bispecific diabodies, as opposed to bispecific whole antibodies, may also be useful because they can be readily constructed and expressed in *E. coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

A "binding domain" is the part of a specific binding member which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where the binding member is an antibody or antigen-binding fragment thereof, the binding domain may be a CDR. Where an antigen is large, an antibody may only bind to a particular part of
5 the antigen, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. An antigen binding domain may comprise an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

"Specific" is generally used to refer to the situation in which one member of a specific
10 binding pair will not show any significant binding to molecules other than its specific binding partner(s), and, e.g., has less than about 30%, preferably 20%, 10%, or 1% cross reactivity with any other molecule. The term is also applicable where e.g., an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case, the specific binding member carrying the antigen binding
15 domain will be able to bind to the various antigens carrying the epitope. Specific binding members of the invention may be capable of binding specifically to Le^y in the sense that there is no detectable binding to any other antigen (such as any other glycan) when binding is tested according to the protocol set out in "Glycome Analysis" in the Examples herein.

"Isolated" refers to the state in which specific binding members of the invention or nucleic acid encoding such binding members will preferably be, in accordance with the present invention. Members and nucleic acid will generally be free or substantially free of material with which they are naturally associated such as other polypeptides or nucleic acids with which they are found in their natural environment, or the
20 environment in which they are prepared (e.g., cell culture) when such preparation is by recombinant DNA technology practised *in vitro* or *in vivo*. Specific binding members and nucleic acid may be formulated with diluents or adjuvants and still for practical purposes be isolated – for example, the members will normally be mixed with gelatin or other carriers
25 if used to coat microtitre plates for use in immunoassays, or will be mixed with pharmaceutically acceptable carriers or diluents when used in diagnosis or therapy. Specific binding members may be glycosylated, either naturally or by systems of heterologous eukaryotic cells, or they may be (for example if produced by expression in a prokaryotic cell) unglycosylated.

By "substantially as set out" it is meant that the amino acid sequence(s) of the invention will be either identical or highly homologous to the amino acid sequence(s) referred to. By "highly homologous" it is contemplated that there may be from 1 to 5, from 1 to 4, from 1 to 3, 2 or 1 substitutions may be made in the sequence.

40 The invention also includes within its scope polypeptides having the amino acid sequence

as set out in Figure 1, 2 or 3, polynucleotides having the nucleic acid sequences as set out in Figure 1 or 2 and sequences having substantial identity thereto, for example at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identity thereto. The percent identity of two amino acid sequences or of two nucleic acid sequences is generally determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the first sequence for best alignment with the second sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences that results in the highest percent identity. The percent identity is determined by comparing the number of identical amino acid residues or nucleotides within the sequences (*i.e.*, % identity = number of identical positions/total number of positions x100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul, 1990 (Karlin and Altschul 1990), modified as in Karlin and Altschul, 1993 (Karlin and Altschul 1993). The NBLAST and XBLAST programs of Altschul *et al.*, 1990 (Altschul, Gish *et al.* 1990) have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, word length = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, word length = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilised as described in Altschul *et al.*, 1997 (Altschul, Madden *et al.* 1997). Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another example of a mathematical algorithm utilised for the comparison of sequences is the algorithm of Myers and Miller, 1989 (Myers and Miller 1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torelli and Robotti, 1994 (Torelli and Robotti 1994); and FASTA described in Pearson and Lipman, 1988 (Pearson and Lipman 1988). Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

Isolated specific binding members of the present invention are capable of binding to a Le^y carbohydrate, which may be a Le^y ceramide or may be on a protein moiety. The binding domains, comprising the amino acid sequences substantially as set out as residues 105 to 116 (CDRH3) of Figure 1a and 1b and 105 to 113 of Figure 1c, may be carried in a

structure which allows the binding of these regions to a Le^y carbohydrate.

The structure for carrying the binding domains of the invention will generally be of an antibody heavy or light chain sequence or substantial portion thereof in which the binding domains are located at locations corresponding to the CDR3 region of naturally-occurring VH and VL antibody variable domains encoded by rearranged immunoglobulin genes. The structures and locations of immunoglobulin variable domains may be determined by reference to <http://www.imgt.org/>. The amino acid sequence substantially as set out as residues 105 to 116 of Figure 1a and 1b may be carried as the CDR3 in a human heavy chain variable domain or a substantial portion thereof, and the amino acid sequence substantially as set out as residues 105 to 113 of Figure 1c may be carried as the CDR3 in a human light chain variable domain or a substantial portion thereof.

The variable domains may be derived from any germline or rearranged human variable domain, or may be a synthetic variable domain based on consensus sequences of known human variable domains. The CDR3-derived sequences of the invention may be introduced into a repertoire of variable domains lacking CDR3 regions, using recombinant DNA technology. For example, Marks *et al.*, 1992 (Marks, Griffiths *et al.* 1992) describe methods of producing repertoires of antibody variable domains in which consensus primers directed at or adjacent to the 5' end of the variable domain area are used in conjunction with consensus primers to the third framework region of human VH genes to provide a repertoire of VH variable domains lacking a CDR3. Marks *et al.*, 1992 (Marks, Griffiths *et al.* 1992) further describe how this repertoire may be combined with a CDR3 of a particular antibody. Using analogous techniques, the CDR3-derived sequences of the present invention may be shuffled with repertoires of VH or VL domains lacking a CDR3, and the shuffled complete VH or VL domains combined with a cognate VL or VH domain to provide specific binding members of the invention. The repertoire may then be displayed in a suitable host system such as the phage display system of WO92/01047 so that suitable specific binding members may be selected. A repertoire may consist of from anything from 10⁴ individual members upwards, for example from 10⁶ to 10⁸ or 10¹⁰ members.

Analogous shuffling or combinatorial techniques are also disclosed by Stemmer, 1994 (Stemmer 1994) who describes the technique in relation to a beta-lactamase gene but observes that the approach may be used for the generation of antibodies. A further alternative is to generate novel VH or VL regions carrying the CDR3-derived sequences of the invention using random mutagenesis of, for example, the FG27VH or VL genes to generate mutations within the entire variable domain. Such a technique is described by Gram *et al.*, 1992 (Gram, Marconi *et al.* 1992), who used error-prone PCR.

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Another method which may be used is to direct mutagenesis to CDR regions of VH or VL genes. Such techniques are disclosed by Barbas *et al.*, 1994 (Barbas, Hu et al. 1994) and Schier *et al.*, 1996 (Schier, McCall et al. 1996). A substantial portion of an immunoglobulin variable domain will generally comprise at least the three CDR regions, together with
5 their intervening framework regions. The portion may also include at least about 50% of either or both of the first and fourth framework regions, the 50% being the C-terminal 50% of the first framework region and the N-terminal 50% of the fourth framework region. Additional residues at the N- terminal or C-terminal end of the substantial part of the
10 variable domain may be those not normally associated with naturally occurring variable domain regions. For example, construction of specific binding members of the present invention made by recombinant DNA techniques may result in the introduction of N- or C-terminal residues encoded by linkers introduced to facilitate cloning or other manipulation steps, including the introduction of linkers to join variable domains of the invention to further protein sequences including immunoglobulin heavy chains, other variable domains
15 (for example in the production of diabodies) or protein labels as discussed in more detail below.

The invention provides specific binding members comprising a pair of binding domains based on the amino acid sequences for the VL and VH regions substantially as set out
20 in Figure 1, *i.e.*, amino acids 1 to 127 (VH) of Figure 1a or 1b and amino acids 1 to 124 (VL) of Figure 1c. Single binding domains based on either of these sequences form further aspects of the invention. In the case of the binding domains based on the amino acid sequence for the VH region substantially set out in Figure 1a and 1b, such binding domains may be used as targeting agents since it is known that immunoglobulin VH
25 domains are capable of binding target antigens in a specific manner. In the case of either of the single chain specific binding domains, these domains may be used to screen for complementary domains capable of forming a two-domain specific binding member which has *in vivo* properties as good as or equal to the FG27 antibodies disclosed herein.

30 This may be achieved by phage display screening methods using the so-called hierarchical dual combinatorial approach as disclosed in WO92/01047 in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain specific binding member is selected in accordance with phage display techniques such as those
35 described in that reference. This technique is also disclosed in Marks *et al.*, 1992 (Marks, Griffiths et al. 1992).

Specific binding members of the present invention may further comprise antibody constant regions or parts thereof. For example, specific binding members based on the VL region
40 shown in Figure 1c may be attached at their C-terminal end to antibody light chain constant

domains Similarly, specific binding members based on VH region shown in Figure 1a and 1b may be attached at their C-terminal end to all or part of an immunoglobulin heavy chain derived from any antibody isotype, e.g., IgG, IgA, IgE and IgM and any of the isotype sub-classes, particularly IgG1, IgG2 and IgG4.

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Specific binding members of the present invention can be used in methods of diagnosis and treatment of tumours in human or animal subjects.

When used in diagnosis, specific binding members of the invention may be labelled with a detectable label, for example a radiolabel such as ^{131}I or ^{99}Tc , which may be attached to specific binding members of the invention using conventional chemistry known in the art of antibody imaging. Labels also include enzyme labels such as horseradish peroxidase. Labels further include chemical moieties such as biotin which may be detected via binding to a specific cognate detectable moiety, e.g., labelled avidin.

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Although specific binding members of the invention have in themselves been shown to be effective in killing cancer cells, they may additionally be labelled with a functional label. Functional labels include substances which are designed to be targeted to the site of cancer to cause destruction thereof. Such functional labels include toxins such as ricin and enzymes such as bacterial carboxypeptidase or nitroreductase, which are capable of converting prodrugs into active drugs. In addition, the specific binding members may be attached or otherwise associated with chemotherapeutic or cytotoxic agents, such as maytansines (DM1 and DM4), onides, auristatins, calicheamicin, duocamycin, doxorubicin or radiolabels, such as ^{90}Y or ^{131}I .

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Furthermore, the specific binding members of the present invention may be administered alone or in combination with other treatments, either simultaneously or sequentially, dependent upon the condition to be treated. Thus, the present invention further provides products containing a specific binding member of the present invention and an active agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a tumour. Active agents may include chemotherapeutic or cytotoxic agents including, 5-Fluorouracil, cisplatin, Mitomycin C, oxaliplatin and tamoxifen, which may operate synergistically with the binding members of the present invention. Other active agents may include suitable doses of pain relief drugs such as non-steroidal anti-inflammatory drugs (e.g., aspirin, paracetamol, ibuprofen or ketoprofen) or opiates such as morphine, or anti-emetics.

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Whilst not wishing to be bound by theory, the ability of the binding members of the invention to synergise with an active agent to enhance tumour killing may not be due to immune effector mechanisms but rather may be a direct consequence of the binding

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member binding to cell surface bound Le^y glycans. Cancer immunotherapy, involving antibodies to immune checkpoint molecules, have shown effectiveness to various malignances and in combinations with different immune-oncology treatment modalities.

- 5 Specific binding members of the present invention will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the specific binding member. The pharmaceutical composition may comprise, in addition to active ingredient, a pharmaceutically acceptable excipient, diluent, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should
- 10 be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g., intravenous. It is envisaged that injections will be the primary route for therapeutic administration of the compositions although delivery through a catheter or other surgical tubing is also used. Some suitable routes of administration
- 15 include intravenous, subcutaneous, intraperitoneal and intramuscular administration. Liquid formulations may be utilised after reconstitution from powder formulations.

- For intravenous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has
- 20 suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

- 25 Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or
- 30 polyethylene glycol may be included. Where the formulation is a liquid it may be, for example, a physiologic salt solution containing non-phosphate buffer at pH 6.8-7.6, or a lyophilised powder.

- The composition may also be administered via microspheres, liposomes, other
- 35 microparticulate delivery systems or sustained release formulations placed in certain tissues including blood. Suitable examples of sustained release carriers include semi-permeable polymer matrices in the form of shared articles, e.g., suppositories or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (US Patent No. 3, 773, 919; EP-A-0058481) copolymers of L-glutamic acid and
- 40 gamma ethyl-L-glutamate (Sidman, Steber et al. 1983), poly (2-hydroxyethyl-methacrylate).

- Liposomes containing the polypeptides are prepared by well-known methods: DE 3,218, 121A; (Eppstein, Marsh et al. 1985); (Hwang, Luk et al. 1980); EP-A-0052522; EP-A-0036676; EP-A-0088046; EP-A-0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos 4,485,045 and 4,544,545. Ordinarily, the liposomes are of the small (about 200-800
- 5 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rate of the polypeptide leakage. The composition may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells.
- 10 The compositions are preferably administered to an individual in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, *e.g.*, decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors,
- 15 and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. The compositions of the invention are particularly relevant to the treatment of existing tumours, especially cancer, and in the prevention of the recurrence of such conditions after initial treatment or surgery. Examples of the techniques and protocols
- 20 mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980 (Remington 1980).

The optimal dose can be determined by physicians based on a number of parameters including, for example, age, sex, weight, severity of the condition being treated, the

25 active ingredient being administered and the route of administration. In general, a serum concentration of polypeptides and antibodies that permits saturation of receptors is desirable. A concentration in excess of approximately 0.1nM is normally sufficient. For example, a dose of 100mg/m² of antibody provides a serum concentration of approximately 20nM for approximately eight days.

30 As a rough guideline, doses of antibodies may be given weekly in amounts of 10-300mg/m². Equivalent doses of antibody fragments should be used at more frequent intervals in order to maintain a serum level in excess of the concentration that permits saturation of the Le^y carbohydrate. The dose of the composition will be dependent upon

35 the properties of the binding member, *e.g.*, its binding activity and *in vivo* plasma half-life, the concentration of the polypeptide in the formulation, the administration route, the site and rate of dosage, the clinical tolerance of the patient involved, the pathological condition afflicting the patient and the like, as is well within the skill of the physician. For example, doses of 300µg of antibody per patient per administration are preferred, although

40 dosages may range from about 10µg to 6mg per dose. Different dosages are utilised

during a series of sequential inoculations; the practitioner may administer an initial inoculation and then boost with relatively smaller doses of antibody.

5 This invention is also directed to optimise immunisation schedules for enhancing a protective immune response against cancer.

The binding members of the present invention may be generated wholly or partly by chemical synthesis. The binding members can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, 1984 (Stewart and Young 1984), in M. Bodanzsky and A. Bodanzsky, 1984 (Bodanzsky and Bodanzsky 1984) or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g., by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

20 Another convenient way of producing a binding member according to the present invention is to express the nucleic acid encoding it, by use of nucleic acid in an expression system.

The present invention further provides an isolated nucleic acid encoding a specific binding member of the present invention. Nucleic acid includes DNA and RNA. In a preferred aspect, the present invention provides a nucleic acid which codes for a specific binding member of the invention as defined above. Examples of such nucleic acid are shown in Figure 1 and 2. The skilled person will be able to determine substitutions, deletions and/or additions to such nucleic acids which will still provide a specific binding member of the present invention.

30 The present invention also provides constructs in the form of plasmids, vectors, transcription or expression cassettes which comprise at least one nucleic acid as described above. The present invention also provides a recombinant host cell which comprises one or more constructs as above. As mentioned, a nucleic acid encoding a specific binding member of the invention forms an aspect of the present invention, as does a method of production of the specific binding member which method comprises expression from encoding nucleic acid. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression, a specific binding member may be isolated and/or purified using any suitable technique, then used as appropriate.

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Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is *E. coli*. The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, 1991 (Plückthun 1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a specific binding member, see for recent review, for example Reff, 1993 (Reff 1993); Trill *et al.*, 1995 (Trill, Shatzman *et al.* 1995).

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral *e.g.*, 'phage, or phagemid, as appropriate. For further details see, for example, Sambrook *et al.*, 1989 (Sambrook 1989). Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Ausubel *et al.*, 1992 (Ausubel 1992).

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, *e.g.*, vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction may be followed by causing or allowing expression from the nucleic acid, *e.g.*, by culturing host cells under conditions for expression of the gene.

The nucleic acid of the invention may be integrated into the genome (*e.g.*, chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

The present invention also provides a method which comprises using a construct as stated above in an expression system in order to express a specific binding member or polypeptide as above.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

- 5 The inventors have unexpectedly found that a family of mAbs FG27 recognise Le^y containing glycans induced non-apoptotic direct cell death without immune effector cells. In one aspect, of the present invention it provides drugs which bind to Le^y containing glycan and induce non-apoptotic cell death.
- 10 FG27 mAbs induced membrane damage to cells resulting in cell clumping, loss of microvilli, uptake of small molecular weight dyes and pore formation. Over time cells developed larger pores and lysed in a mechanism similar to oncosis. This is similar to a number of mAbs recognising other glycans (Hellstrom, Garrigues et al. 1990, Chou, Takematsu et al. 1998, Zhong, Manzi et al. 2001, Alvarez-Rueda, Leprieur et al. 2007, Loo,
- 15 Pryer et al. 2007, Zhang, Zhang et al. 2010). The FG27 mAbs also exhibited potent *in vitro* cytotoxic activity through ADCC and CDC. The administration of FG27 (0.1mg intravenous (i.v.) twice a week for 9 weeks) to mice with established metastases in the liver and peritoneal cavity resulted in complete tumour eradication and cure of ~30% of the mice. The potential of FG27 mAbs in eradicating well-established tumours without
- 20 concomitant chemotherapy indicates their potential as monotherapeutic agents for the treatment of Le^y expressing human solid tumours.

The invention will now be described further in the following non-limiting examples and accompanying drawings, in which:

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- Figure 1a:** Complete amino acid and nucleotide sequence of the mouse FG27.10 IgG3 heavy chain. Numbers refer to the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009). **Figure 1b:** Complete amino acid and nucleotide sequence of mouse FG27.18 IgG1 heavy chain. Numbers refer to the
- 30 standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009). **Figure 1c:** Complete amino acid and nucleotide sequence of mouse FG27.10 and FG27.18 kappa chain. Numbers refer to the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009).
- 35 **Figure 2a:** Complete amino acid and nucleotide sequence of mouse FG27 heavy chain variable region chimerised to human IgG1 heavy chain constant region. Numbers refer to the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009). **Figure 2b:** Complete amino acid and nucleotide sequence of mouse FG27 kappa chain variable region chimerised to human kappa chain constant region. Numbers
- 40 refer to the standardised IMGT system for the numbering of antibody sequences (Lefranc,

Giudicelli et al. 2009).

Figure 3a: Complete amino acid and nucleotide sequence of the humanised FG27 heavy chain variable region. Numbers refer to the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009). **Figure 3b:** Complete amino acid and nucleotide sequence of humanised FG27 kappa chain variable region. Numbers refer to the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009).

Figure 4: Binding of FG27.10 and FG27.18 to ST16, HUVEC and PBMCs. The negative control for this assay was NSO supernatant for ST16 and HUVEC, or cell alone for the PBMCs and the positive control was either the anti-sialyl-di Le^a mAb, 505/4, or W6/32 which recognises HLA.

Figure 5: Binding of FG27.10 and FG27.18 to glycolipid extracts from colorectal (C170), ovarian (OVCAR-3, OVCAR-4, OVCA433, OAW28, OAW42) and gastric cell lines (ST16, MKN45, AGS). Glycolipid extracted from a range of cancer cell lines was plated on ELISA plates. Cells and glycolipid were then incubated with FG27.10 or FG27.18 supernatant. Binding to glycolipid was probed with anti-IgG-HRP/TMB.

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Figure 6: Binding of FG27.10 to HSA coupled Lewis antigens (a, b, x and y) as assessed by ELISA. 505/4 (anti-sialyl-di-Le^a), FG88.2 (Le^{a-x}), FG88.7 (Le^{a-x}) and 225-Le (Le^b) were included as positive controls and mouse HRPO as the negative. Antibody activity was measured by absorbance at 405nm.

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Figure 7: Binding of FG27.10 and FG27.18 were screened against The Consortium for Functional Glycomics glycan array which is composed of 610 mammalian glycan targets. The fine specificity between a) FG27.10, b) FG27.18, c) 692 (Le^{y/b}), and d) BR96 (Le^{y/x}) are compared; where a=Le^a, b=Le^b, y=Le^y, x=Le^x, D- = Di, T- = Tri, S- = sialyl, Ex- = extended, φ denotes a mannose containing glycan, and y-x= Le^y-Le^x. Note due to the mAbs being screened on different versions of the glycan array, the glycan numbers may not be the same for each mAb.

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Figure 8: Binding of FG27 to Le^y attached to total proteins or lipids extracted from a panel of tumour cell lines. Western blot detection of FG27 binding to Le^y associated with total glycoproteins or glycolipids extracted from the tumour cell lines, AGS, HCT15, OVCAR3, MCF7 and H322 cell lysates (1x10⁵ cells equivalent).

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Figure 9: Comparison of anti-Le^y and Le^b mAbs on a range of cell lines. SC101 (692/29) and BR96 were titrated across a range of cell lines (ST16, HT29, C170, LoVo,

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Colo201, OVCAR-3, AGS) at 0.1-30µg/ml, with FG27 supernatant being diluted from neat to 1:30. Binding was probed with anti-IgG-FITC and analysed by flow cytometry.

Figure 10: Representative IHC images of normal oesophagus, stomach, duodenum, ileum, colon, pancreas, lung and kidney stained with 692/29 (Le^{y/b}), BR96 (Le^{y/x}) and FG27 (Le^y).

Figure 11a: In vivo anti-tumour activity of FG27. Percentage tumour growth over the duration of the study is shown with the C170HM2 bioluminescence mouse tumour model used to assess the anti-tumour activity of the murine FG27 mAb compared to the vehicle only control (PBS). In this model bioluminescence represents viability. Group n≥9; the study was terminated on day 32. Treatment with FG27 resulted in a significant reduction in bioluminescent tumour burden by the last day of the study compared to the vehicle control (p=0.014). **Figure 11b:** Injection site tumour burden as assessed by BLI, showing a significant reduction in injection site tumour growth with FG27 treatment (p=0.05).

Figure 12: In vivo anti-tumour activity of FG27; Percentage tumour growth is shown with the C170HM2 bioluminescence mouse tumour model used to assess the anti-tumour activity of FG27 compared to the positive control mAbs and vehicle only control (PBS). Bioluminescence represents tumour cell viability in this model. Group n≥ 8; the treatment with FG27 produced a significant reduction in percentage tumour growth by day 51 (p=0.009) compared to the treatment control. **Figure 12b:** Analysis by Log Rank Mantel-Cox test demonstrates significant survival in the FG27 (p=0.035) treatment group compared to the vehicle only control. Treatment was stopped at day 120.

Figure 13a: Amino acid and nucleotide sequence of mouse FG27 heavy chain variable region compared to BR96, H18A, Hu3S193 and SC101 using the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009). **Figure 13b:** Amino acid and nucleotide sequence of mouse FG27 kappa chain variable region compared to BR96, SC101, H18A, Hu3S193 using the standardised IMGT system for the numbering of antibody sequences (Lefranc, Giudicelli et al. 2009).

Figure 14: Binding, as assessed by indirect immunofluorescence and flow cytometric analysis, of chimeric IgG1 (5µg/ml) and IgG2 (5µg/ml) mAbs to the Le^y expressing tumour cell line, AGS are compared to the FG27.10 (5µg/ml) and FG27.18 (5µg/ml) murine versions of the antibody. The anti-HLA mAb, W6/32 (1/100) was included as a positive control.

Figure 15a: PI uptake of ST16 cells mediated by the mouse IgG3 mAb FG27.10 induced potent uptake of PI even at 4°C but the IgG1 variant FG27.18 did not. **Figure 15b:**

PI uptake mediated by FG27.10 and FG27.18 on a panel of tumour cell lines (AGS, Colo201, C170, C170HM2, MCF7, OVCA4, LoVo, MKN45, 791T and Skov3). **Figure 15c:** PI uptake mediated by FG27.10, FG27.18, CH27 IgG2, CH27 IgG1 on C170 cells.

- 5 **Figure 16:** FG27.10, FG27.18 and CH27 internalisation data over a range of concentrations (0.01nM to 30nM) on a panel of cancer cell lines, AGS, MCF7, H322 and C170. Cellular internalisation of the mAbs was observed using 75ng/well saporin-conjugated goat antibody (F'ab fragment) to mouse (ATSBio, IT#48) or human IgG (ZAP) and assessed by inhibition of thymidine uptake (added for the last 24hrs of the incubation).
- 10 Control wells consisted of cells incubated with culture medium only, with the irrelevant positive control IgG3 mAb FG88 and with secondary conjugate only.

- Figure 17a:** Example of FG27.10 mIgG3 mediating ADCC/CDC. Target cells were incubated with PBMCs isolated from human blood (ADCC), or complement (CDC) with either FG27.10 supernatant or irrelevant IgG. Error bars representing standard deviation of triplicate wells are present but may be obscured by data points. **Figure 17b:** ADCC of MCF7 tumour cell line on incubation with FG27.10, FG27.18, 692/29, CH27 IgG1 and CH27 IgG2. These are the results from one experiment but are representative of 2 repeats. **Figure 17c:** Mean percentage killing by CDC of the ST16 tumour cell line following incubation with FG27.10, FG27.18, 692/29, CH27 IgG1 and CH27 IgG2 with human serum after 4hrs. Herceptin was included as a control. The assay was performed at serial dilutions 3, 1, 0.3 and 0.1µg/ml and each dilution was incubated in triplicate.
- 20 **Figure 17d:** Mean percentage killing by CDC of AGS cells following incubation with FG27.10, 692/29, CH27 IgG1 and CH27 IgG2 with human serum after 4hrs. The assay was performed at serial dilutions 3, 1, 0.3 and 0.1µg/ml and each dilution was incubated in triplicate.
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- Figure 18:** FG27 Heavy Chain Gibbs Analysis I: Sequence Identities. Structurally important residues are highlighted: Proline residues outlined in White, Cysteine residues outlined in Black, and Asparagine residues in Grey.
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Figure 19: FG27 Heavy Chain Gibbs Analysis II: Identity and Similarity Scores, 4Å Proximity Residues and CDR LoopLengths. Cysteine residues are outlined in White.

- 35 **Figure 20:** Human Heavy Chain Donor Sequence Germ Line Analysis.

- Figure 21:** FG27 Heavy Chain Humanisation Strategy Structurally important residues are highlighted: Proline residues are outlined in White, Cysteine residues are outlined in Black and Asparagine residues in Grey. Residues outlined in Grey and in italics indicate back-translations to the Mouse Residue.
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Figure 22: 27 Kappa Light Chain Gibbs Analysis I: Sequence Identities. Structurally important residues are highlighted: Proline residues are outlined in White, Cysteine residues are outlined in Black, and Asparagine residues in Grey.

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Figure 23: FG27 Kappa Light Chain Gibbs Analysis II: Identity and Similarity Scores, 4Å Proximity Residues and CDR Loop Lengths.

Figure 24: Human Kappa Light Chain Donor Sequence Germ Line Analysis.

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Figure 25: FG27 Kappa Light Chain Humanisation Strategy. Structurally important residues are highlighted: Proline residues are outlined in White, and Cysteine residues are outlined in Black. Residues in Bold indicate back-translations to the Mouse Residue.

15 **Figure 26:** Comparison of antibody binding to LewisY-HSA antigen for combinations of antibody expressed as LewRHA with LewRKA, LewRKB, LewRKC or LewRKD and LewRHB with LewRKA, LewRKB, LewRKC or LewRKD, including cFG27 positive control antibody and c1210 negative control antibody.

20 **Figure 27.** The thermal stability of LewRHA with LewRKA, LewRKB, LewRKC or LewRKD and LewRHB with LewRKA, LewRKB, LewRKC or LewRKD, including cFG27 positive control antibody.

Figure 28. The thermal shift analysis of Lewis^y FG27 humanised variant hLewAB, hLewAC, hLewAD, hLewBC and control mouse FG27.

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Figure 29. BiaCore analysis of antibody affinity of FG27 humanised variant hLewAB, hLewAC, hLewAD, hLewBC and control mouse FG27.

30 **Figure 30.** Non-specific Protein-Protein Interactions (CIC) as measured by Cross-Interaction Chromatography on FG27 humanised variant hLewAB, hLewAC, hLewAD, hLewBC and control mouse FG27.

Figure 31. Binding of FG27.10, FG27.18, ch27IgG1 and humanised variant hLewAC to a panel of cell lines.

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Figure 32: Significantly increased proliferation inhibition on MCF7 (Panel i) and AGS (Panel ii) by i27G1 compared to 27hlgG1. Significantly increased functional affinity (SPR) by i27G1 compared to 27hlgG1 (Panel iii). i27G1 maintains equivalent ADCC (Panel iv) as well as CDC activity (panel v) on MCF7. Significance versus parental constructs was deduced from two-

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way ANOVA (direct cytotoxicity) or one-way ANOVA (functional affinity) with Dunnett's corrections for multiple comparisons.

Figure 33: Amino acid and nucleic acid sequences of the respective light and heavy chains of the 'i27G1' antibody.

EXAMPLES

Methods

- 10 **Binding to tumour cell lines:** 1×10^5 cancer cells were incubated with 50µl of primary antibodies at 4°C for 1hr. Cells were washed with 200µl of RPMI 10% new born calf serum (NBCS: Sigma, Poole, UK) and spun at 1,000rpm for 5mins. Supernatant was discarded and 50µl of FITC conjugated anti-mouse IgG Fc specific mAb (Sigma; 1/100 in RPMI 10% NBCS) was used as secondary antibody. Cells were incubated at 4°C in dark
- 15 for 1hr then washed with 200µl RPMI 10% NBCS and spun at 1,000rpm for 5mins. After discarding supernatant, 0.4% formaldehyde was used to fix the cells. Samples were analysed on a Beckman coulter FC-500 flow cytometer (Beckman Coulter, High Wycombe, UK). To analyse and plot raw data, WinMDI 2.9 software was used.
- 20 **Binding to blood:** 50µl of healthy donor blood was incubated with 50µl primary antibody at 4°C for 1hr. The blood was washed with 150µl of RPMI 10% NBCS and spun at 1,000rpm for 5mins. Supernatant was discarded and 50µl FITC conjugated anti- mouse IgG Fc specific mAb (1/100 in RPMI 10% NBCS) was used as the secondary antibody. Cells were incubated at 4°C in the dark for 1hr then washed with 150µl RPMI 10% NBCS
- 25 and spun at 1,000rpm for 5mins. After discarding the supernatant, 50µl/well Cal-Lyse (Invitrogen, Paisley, UK) was used followed by 500µl/well distilled water to lyse red blood cells. The blood was subsequently spun at 1,000rpm for 5mins. Supernatant was discarded and 0.4% formaldehyde was used to fix the cells. Samples were analysed on a FC-500 flow cytometer (Beckman Coulter). To analyse and plot raw data, WinMDI 2.9 software
- 30 was used.

- Plasma membrane glycolipid extraction:** A cell pellets (5×10^7 cells) were resuspended in 500µl of Mannitol/HEPES buffer (50mM Mannitol, 5mM HEPES, pH7.2, both Sigma) and passed through 3 needles (23G, 25G, 27G) each with 30 pulses. 5µl of
- 35 1M CaCl_2 was added to the cells and passed through 3 needles each with 30 pulses as above. Sheared cells were incubated on ice for 20mins then spun at 3,000g for 15mins at room temperature (RT). Supernatant was collected and spun at 48,000g for 30mins at 4°C and the supernatant was discarded. The pellet was resuspended in 1ml methanol followed by 1ml chloroform and incubated with rolling for 30mins at RT. The sample was then spun
- 40 at 1,200g for 10mins to remove precipitated protein. The supernatant, containing plasma

membrane glycolipids, was collected and stored at -20°C.

Glycome analysis: To clarify the fine specificities of the FG27 mAbs, the antibodies were FITC labelled and sent to the Consortium for Functional Glycomics (<http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml>) where they were screened against ≥600 natural and synthetic glycans. Briefly, synthetic and mammalian glycans with amino linkers were printed onto N-hydroxysuccinimide (NHS)-activated glass microscope slides, forming amide linkages. This work was done by Core H of the CFG at Emory University. FG27 was tested at 1 µg/ml in PBS at room temperature. Briefly, antibody samples were applied to the printed surface of the microarray and incubated in a humidified chamber for 1 h. The slide was then rinsed 4 times with PBS, followed by addition of fluorescently labeled (Alexa Fluor 488) anti-rabbit IgG, and was incubated for 1 h. The slide was then rinsed 4 times in PBS, and fluorescence was measured with a Perkin-Elmer microarray XL4000 scanner and analyzed using Imogene software (BioDiscovery).

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Further details of the protocol used is set out below:

Glycan Binding Assay with Unlabelled Monoclonal Antibody

1. Introduction:

20 1.1. Core H primary objective is to determine the binding specificity of Glycan Binding Proteins (GBPs) and various organisms submitted by investigators using the printed glycan microarray.

2. Reference:

2.1. www.functionalglycomics.org

25 3. Materials needed:

- 3.1. Glycan printed slides (*Core D*), printed on the side of the slide with the white etched bar code and black marks- **DO NOT TOUCH THIS AREA**
- 3.2. Cover slips (Fisher scientific, 12-545F)
- 3.3. Humidified Slide processing chambers (Fisher scientific, NC9091416), or homemade system using Petri Dish, with wet paper towels in the bottom of the chamber
- 30 3.4. 100 ml Coplin jars for washing slides
- 3.5. Tris-HCl (Fisher scientific, BP152-1)
- 3.6. NaCl (Fisher scientific, S271-3)
- 3.7. CaCl₂ (Fisher scientific, C79-500)
- 35 3.8. MgCl₂ (Fisher scientific, BP214-500)
- 3.9. Potassium Phosphate Monobasic (Fisher scientific, P285-3)
- 3.10. dH₂O
- 3.11. Cyanine 5- Streptavidin (ZYMED 43-4316)
- 3.12. Appropriate secondary antibody, fluorescently labeled if available
- 40 3.13. BSA (Fisher scientific, Bp1600-100)

- 3.14. Tween-20 (EMD Biosciences, 655205)
- 3.15. Sodium Azide (fisher scientific, S227-500)
- 3.16. ProScanArray Scanner (Perkin Elmer)

4. Buffers:

- 5 4.1. TSM= 20mM Tris-HCl, pH 7.4 150mM NaCl, 2mM CaCl₂, 2mM MgCl₂
- 4.2. TSM Wash Buffer (TSMW)= TSM Buffer + 0.05% Tween-20
- 4.3. TSM Binding Buffer (TSMBB)= TSM buffer +0.05% Tween 20 + 1% BSA

5. Protocol:

- 10 5.1. Make working stocks of washing buffers (TSM, TSM Wash Buffer, and H₂O) or collect reagents and bring to room temperature if they have been in the refrigerator.
 - 5.1.1. Buffer (A) TSM- 20mM Tris-HCl, pH 7.4 150mM NaCl, 2mM CaCl₂, 2mM MgCl₂
 - 5.1.2. Buffer (B) TSM Wash Buffer (TSMW) - TSM Buffer + 0.05% Tween-20
 - 5.1.3. Buffer (C) TSM Binding Buffer (TSMBB) – TSM buffer +0.05% Tween 20 + 1% BSA
 - 5.1.4. dH₂O
- 15 5.2. Prepare 100 µl of sample by diluting antibody in TSMBB or appropriate Binding Buffer based on properties of Antibody to a final concentration of 5-50ug/ml or an appropriate concentration required for the analysis.
- 5.3. Remove slide(s) from desiccator and label slide with sample name near barcode, outside of the black marks.
- 20 5.4. Hydrate the slide by placing in a glass Coplin staining jar containing 100 ml of TSMW for 5 min.
- 5.5. Remove excess liquid from slide by setting the slide upright to drain the liquid off.
- 5.6. Carefully apply 70 µl of sample (see 5.2) close to the left edge slide in between the black marks.
- 25 5.7. Slowly place cover slip on slide, trying to avoid the formation of bubbles in the sample under the cover slip. Remove any bubbles by gently tapping the cover slip with a pipette tip if necessary, or slowly lifting one side of the cover slip. Make sure the cover slip is between the black marks.

30

- SDS-PAGE and Western blot analysis:** Briefly, 10⁵ or 10⁶ cell equivalents (cell lysate, plasma membrane lipid extract) were analysed for FG27 binding. Tumour cell plasma membrane lipid extracts and cell lysates were reduced with dithiothreitol (DTT; Pierce Biotechnology, ThermoFisher, Loughborough, UK) and subjected to SDS-PAGE using
- 35 NOVEX 4% to 12% Bis-Tris gels (Invitrogen), and transferred to Hybond-P PVDF membranes (GE Healthcare, Amersham, UK) using 1x transfer buffer (20x, Invitrogen) and 20% (v/v) methanol at 30V for 1hr. Membranes were blocked with 5% (w/v) non-fat dry milk in 0.05% (v/v) Tween-PBS for 1hr then probed with primary antibodies diluted in Tween-PBS, 2% BSA for 1hr. Primary antibody binding was detected using biotin-
- 40 conjugated anti-mouse IgG Fc specific secondary antibody(Sigma; 1/2000 dilution in

Tween-PBS, 2% BSA) for 1hr, and visualised using IRDye 800CW streptavidin (LICOR Biosciences, UK; 1/1000 in Tween-PBS 2% BSA).

- Immunohistochemistry assessment for FG27:** To determine the therapeutic value of FG27, it was screened on gastric, colorectal, ovarian and breast tumour tissue microarrays by immunohistochemistry (IHC).

Methodology: Immunohistochemistry was performed using the standard avidin-biotin peroxidase method. Paraffin embedded tissue sections were placed on a 60°C hot block to melt the paraffin. Tissue sections were deparaffinised with xylene and rehydrated through graded alcohol. The sections were then immersed in 500ml of citrate buffer (pH6) and heated for 20mins in a microwave (Whirlpool) to retrieve antigens. Endogenous peroxidase activity was blocked by incubating the tissue sections with endogenous peroxidase solution (Dako Ltd, Ely, UK) for 5mins. Normal swine serum (NSS; Vector Labs, CA, USA; 1/50 PBS) was added to each section for 20mins to block non-specific primary antibody binding. All sections were incubated with Avidin D/Biotin blocking kit (Vector Lab) for 15mins each in order to block non-specific binding of avidin and biotin. The sections were re-blocked with NSS (1/50 PBS) for 5mins. Then tissue sections were incubated with primary antibody at RT for an hr. Anti-beta-2-microglobulin (Dako Ltd; 1/100 in PBS) mAb and PBS alone were used as positive and negative controls respectively. Tissue sections were washed with PBS and incubated with biotinylated goat anti-mouse/rabbit immunoglobulin (Vector Labs; 1/50 in NSS) for 30mins at RT. Tissue sections were washed with PBS and incubated with preformed 1/50 (PBS) streptavidin-biotin/horseradish peroxidase complex (Dako Ltd) for 30mins at RT. 3, 3'-Diaminobenzidine tetra hydrochloride (DAB) was used as a substrate. Each section was incubated twice with 100µl of DAB solution for 5mins. Finally, sections were lightly counterstained with haematoxylin (Sigma-Aldrich, Poole Dorset, UK) before dehydrating in graded alcohols, cleaning by immersing in xylene and mounting the slides with Distyrene, plasticiser, xylene (DPX) mountant (Sigma).

30

Identification of FG27.10 and FG27.18 heavy and light chain variable regions

Cell source and total RNA preparation: Approximately 5×10^6 cells from hybridomas FG27.10 and FG27.18 were taken from tissue culture, washed once in PBS, and the cell pellet treated with 500µl Trizol (Invitrogen). After the cells had been dispersed in the reagent, they were stored at -80°C until RNA was prepared following manufacturer's protocol. RNA concentration and purity were determined by Nanodrop. Prior to cDNA synthesis, RNA was DNase I treated to remove genomic DNA contamination (DNase I recombinant, RNase-free, Roche Diagnostics, Burgess Hill, UK) following manufacturer's recommendations.

40

cDNA synthesis: First-strand cDNA was prepared from 3µg of total RNA using a first-strand cDNA synthesis kit and AMV reverse transcriptase following manufacturer's protocol (Roche Diagnostics). After cDNA synthesis, reverse transcriptase activity was destroyed by incubation at 90°C for 10mins and cDNA stored at -20°C.

5

GAPDH PCR to assess cDNA quality: A PCR was used to assess cDNA quality; primers specific for the mouse GAPDH house-keeping gene (5'-TTAGACCCCCTGGCCAAGG-3' and 5'-CTTACTCCCTTGGAGGCCATG-3') were used with a hot-start Taq polymerase (NEB, Hitchin, UK) for 35 cycles (95°C, 3mins followed by 35 cycles of 94°C/30secs, 55°C/30secs, 72°C/1min; final polishing step of 10mins at 72°C). Amplified products were assessed by agarose gel electrophoresis.

PCR primer design for cloning FG27 variable regions: Primers were designed to amplify the heavy and light chain variable regions based upon the PCR product sequence data. Primers were designed to allow cloning of the relevant chain into unique restriction enzyme sites in the hlgG1/kappa double expression vector pDCOrig-hlgG1. Each 5' primer was targeted to the starting codon and leader peptide of the defined variable region, with a Kozak consensus immediately 5' of the starting codon. Each 3' primer was designed to be complementary to the joining region of the antibody sequence, to maintain reading frame after cloning of the chain, and to preserve the amino acid sequence usually found at the joining region/constant region junction. All primers were purchased from Eurofins MWG.

Heavy chain variable region PCR: Immunoglobulin heavy chain variable region usage was determined using PCR with a previously published set of primers (Jones and Bendig 1991). Previous results using a mouse mAb isotyping test kit (Serotec, Oxford, UK) had indicated that FG27.10 was a mouse IgG3 and FG27.18 a mouse IgG1 antibody. Appropriate constant region reverse primers were therefore used to amplify from the constant regions. PCR amplification was carried out with 12 mouse VH region-specific 5' primers and 3' primers specific for previously determined antibody subclass with a hot-start Taq polymerase for 35 cycles (94°C, 5mins followed by 35 cycles of 94°C/1min, 60°C/1min, 72°C/2mins; final polishing step of 20mins at 72°C). Amplified products were assessed by agarose gel electrophoresis. Positive amplifications resulted for the VH4 primer.

35

Light (κ) chain variable region PCRs: Immunoglobulin light chain variable region usage was determined using PCR with a previously published set of primers (Jones and Bendig 1991). Previous results using a mouse mAb isotyping test kit had indicated that both FG27.10 and FG27.18 used κ light chains. PCR amplification was carried out with mouse Vk region-specific 5' and 3' mouse Cκ specific primers with a hot-start Taq

40

polymerase for 35 cycles (94°C, 5mins followed by 35 cycles of 94°C/1min, 60°C/1min, 72°C/2mins; final polishing step of 20mins at 72°C). Amplification products were assessed by agarose gel electrophoresis. Positive amplifications resulted with the Vk1 and Vk2 primers for both FG27.10 and FG27.18.

5

PCR product purification and sequencing: PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Crawley, UK). The concentration of the resulting DNA was determined by Nanodrop and the purity assessed by agarose gel electrophoresis. PCR products were sequenced using the originating 5' and 3' PCR primers at the University of Nottingham DNA sequencing facility (<http://www.nottingham.ac.uk/life-sciences/facilities/dna-sequencing/index.aspx>). Sequences were analysed (V region identification, junction analysis) using the IMGT database search facility (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=mouse1g). Sequencing indicated that FG27.10 and FG27.18 shared identical heavy and light chain variable regions. Sufficient residual constant region was present in the heavy chain sequences to confirm that FG27.10 was of the mIgG3 subclass and FG27.18 a mIgG1 with the two having resulted from a single splenocyte-NSO fusion event.

Cloning strategy: Direct cloning of the PCR products into the pDCOrig-hlgG1 vector using the restriction sites incorporated into the PCR primers was known to be relatively inefficient from previous Scancell experience. A dual cloning strategy was therefore adopted; the PCR product generated using a proof-reading polymerase was cloned into both pDCOrig-hlgG1 and a TA vector (pCR2.1; Invitrogen) simultaneously, with the TA vector-cloned product acting as an easily expanded backup source of material for cloning should the initial pDCOrig-hlgG1 cloning fail.

FG27.18 heavy/light chain PCR for cloning: PCR amplification was carried out using a proof-reading polymerase (Phusion; NEB) and the cloning primers described above using the FG27.18 cDNA template previously described for 35 cycles (98°C, 3mins followed by 35 cycles of 98°C/30secs, 58°C/30secs, 72°C/45secs; final polishing step of 3mins at 72°C). Successful amplification was confirmed by agarose gel electrophoresis.

Method 1 - Direct light chain cloning: Amplified FG27.18 light chain was digested sequentially with the restriction enzymes BsiWI and BamHI according to manufacturer's instructions (NEB). Vector (pDCOrig-hlgG1, containing V regions from a previously cloned antibody) was simultaneously digested. Vector DNA was agarose gel purified using a QIAquick gel extraction kit (Qiagen) and insert DNA purified using a PCR purification kit. After DNA quantification by Nanodrop, vector DNA was phosphatase treated according to manufacturer's recommendations (Antarctic Phosphatase, NEB) and light chain insert ligated into the vector (T4 DNA ligase, NEB). Ligated DNA was

transformed into chemically competent TOP10F' cells (Invitrogen) and spread on 35µg/ml Zeocin (Invitrogen, Toulouse, France) supplemented LB agar plates which were then incubated overnight at 37°C.

- 5 *Method 2 - TOPO light chain cloning:* Amplified FG27.18 light chain was treated with Taq polymerase (NEB) for 15mins at 72°C to add 'A' overhangs compatible with TA cloning. Treated PCR product was incubated with the TOPO TA vector pCR2.1 (Invitrogen) and transformed into chemically competent TOP10F' cells according to manufacturer's instructions. Transformed bacteria were spread on ampicillin (80µg/ml) supplemented LB
10 agar plates which were then incubated overnight at 37°C. Colonies were grown in liquid culture (LB supplemented with 80µg/ml ampicillin) and plasmid DNA prepared (spin miniprep kit, Qiagen). Presence of an insert was confirmed by sequential digestion with BsiWI and BamHI and agarose gel electrophoresis. Sequencing was carried out on miniprep DNA from colonies using T7 and M13rev primers. The DNA insert from one
15 such colony had the predicted FG27.18 light chain sequence; a 300ml bacterial LB/ampicillin culture was grown overnight and plasmid DNA prepared by maxiprep (plasmid maxi kit, Qiagen). Maxiprep DNA insert was confirmed by sequencing.

- TOPO heavy chain cloning:* Amplified FG27.18 heavy chain was treated with Taq
20 polymerase (NEB) for 15mins at 72°C to add 'A' overhangs. Treated PCR product was incubated with the TOPO TA vector pCR2.1 and transformed into chemically competent TOP10F' cells as above. Transformed bacteria were spread on ampicillin supplemented LB agar plates which were then incubated overnight at 37°C. Colonies were grown in liquid culture (LB/ampicillin) and plasmid DNA prepared (spin miniprep kit). Presence of an
25 insert was confirmed by digestion with HindIII and AfeI and agarose gel electrophoresis. Sequencing was carried out on miniprep DNA from a number of colonies using T7 and M13rev primers. The DNA insert from one such colony had the predicted FG27.18 heavy chain sequence; a 300ml bacterial LB/ampicillin culture was grown overnight and plasmid DNA prepared by maxiprep (plasmid maxi kit, Qiagen).
30 Maxiprep DNA insert was confirmed by sequencing.

- pDCOrig-hlgG1 double expression vector light chain cloning:* The FG27.18 light chain was digested from the TOPO vector pCR2.1 by sequential digestion with BsiWI and BamHI and the 400bp insert DNA agarose gel purified using a QIAquick gel
35 extraction kit (Qiagen) following manufacturer's recommendations. This insert was ligated into previously prepared pDCOrig-hlgG1 vector (see above) and transformed into chemically competent TOP10F' cells. Transformations were spread on 35µg/ml Zeocin supplemented LB agar plates which were then incubated overnight at 37°C.

- 40 Colonies were grown in liquid culture (LB supplemented with 35µg/ml Zeocin) and

plasmid DNA prepared (spin miniprep kit, Qiagen). Sequencing was carried out on miniprep DNA from all colonies using the P6 sequencing primer sited in the human kappa constant region. The DNA insert from a colony had the predicted FG27.18 light chain sequence correctly inserted in pDCOrig-hlgG1; a 300ml bacterial LB/zeocin culture was grown overnight and plasmid DNA prepared by maxiprep (plasmid maxi kit, Qiagen).

pDCOrig-hlgG1 double expression vector heavy chain cloning: The FG27.18 heavy chain insert was digested from the TOPO vector pCR2.1 by digestion with HindIII and AfeI. Vector (pDCOrig-hlgG1-27.18k) containing the FG27.18 kappa light chain (prepared above) was also digested with HindIII and AfeI. The vector DNA was then phosphatase treated according to manufacturer's recommendations (Antarctic Phosphatase, NEB). After agarose gel electrophoresis, the 6.5kb pDCOrig-hlgG1 vector band and 400bp FG27.18H insert band were isolated using a QIAquick gel extraction kit (Qiagen) following manufacturer's recommendations. The insert was ligated into the pDCOrig-hlgG1 vector and transformed into chemically competent TOP10F' cells. Transformations were spread on 35µg/ml Zeocin supplemented LB agar plates which were then incubated overnight at 37°C. Colonies were grown in liquid culture (LB supplemented with 35µg/ml Zeocin) and plasmid DNA prepared (spin miniprep kit, Qiagen). Presence of an insert was confirmed by digestion with HindIII and AfeI and agarose gel electrophoresis. Sequencing was carried out on miniprep DNA from a number of the colonies using the P3rev sequencing primer sited in the human IgG1 constant region. The DNA insert from one of the colonies had the predicted FG27.18 heavy chain sequence correctly inserted in pDCOrig-hlgG1; a 300ml bacterial LB/zeocin culture was grown overnight and plasmid DNA prepared by maxiprep (plasmid maxi kit, Qiagen). Sequencing was used to confirm that both heavy and light chain loci.

Expression, purification and characterisation of the chimeric antibody constructs: The methodology for the expression and purification of chimeric antibody described in the present invention can be achieved using methods well known in the art. Briefly, antibodies can be purified from supernatant collected from transiently, or subsequently stable, transfected cells by protein A or protein G affinity chromatography based on standard protocols, for example Sambrook *et al.*, 2001 (Sambrook and Russell 2001).

QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene)

Prepare the reaction(s) as indicated below:

- 5µl of 10× reaction buffer
- 0.12µl (25ng) of RHA or RKA template
- 1.3µl (125ng) of oligonucleotide mutation primer For
- 1.3µl (125ng) of oligonucleotide mutation primer Rev 1µl of dNTPmix
- 1.5µl of QuikSolution reagent ddH₂O to a final volume of 50µl

1µl of QuikChange Lightning Enzyme

1. Cycle each reaction using the cycling parameters outlined in the following table:

Cycle step	3-step protocol		Cycles
	Time	Temp.	
Initial denaturation	95°C	2mins	1
Denaturation Temp.	95°C	20secs	18
Annealing	60°C	10secs	
Extension	68°C	3mins	
Final extension	68°C	5mins hold	1
	4°C		

- 5 2. Add 2µl of the *Dpn* I restriction enzyme
3. Gently and thoroughly mix each reaction, microcentrifuge briefly, then immediately incubate at 37°C for 5mins to digest the parental dsDNA
4. Transform 2µl of the *Dpn* I-treated DNA from each reaction into separate 45µl (+ 2µl β-ME) aliquots of XL10-Gold ultracompetent cells (see *Transformation of TOP10™ E. coli*).
- 10 5. Screen colonies using the Phusion method, miniprep and sequence to check for the correct mutation.

Q5® Site-Directed Mutagenesis Kit (NEB Protocol):

1. Assemble the following reagents in a thin-walled PCR tube:

Reagent	Volume (for 25µl final reaction volume)
Q5 Hot Start High-Fidelity 2X Master Mix	12.5µl
10µM Forward Primer	1.25µl
10µM Reverse Primer	1.25µl
Template DNA (1–25ng/µl)	1µl
Nuclease-free water	9µl

15

20

Cycle

Cycle step	3-step protocol		Cycles
	Time	Temp.	
Initial denaturation	98°C	30secs	1
Denaturation Temp.	98°C	10secs	25
Annealing Extension	69°C	30secs	
	72°C	180secs	
Final extension	72°C	2mins hold	1
	4°C		

Kinase, Ligase & DpnI (KLD) Treatment

- 5
 2. Add the following reagents and incubate for 5mins at RT: 1µl of PCR product
5µl 2X KLD Reaction Buffer 1µl 10X KLD Enzyme Mix
3µl Nuclease-free Water
 3. Transform 5µl of the KLD mix from each reaction into separate 50µl of NEB 5-alpha
- 10
 - Competent *E. coli* Carefully flick the tube 4-5 times to mix. Do not vortex.
 4. Place the mixture on ice for 30mins.
 5. Heat shock at 42°C for 30secs. Place on ice for 5mins.
 6. Pipette 950µl of RT SOC into the mixture.
 7. Incubate at 37°C for 60mins with shaking (250rpm).
- 15
 8. Mix the cells thoroughly by flicking the tube and inverting, then spread 50µl onto a kanamycin plate and incubate overnight at 37°C.
 9. Screen colonies using the GoTaq Green method, miniprep and sequence to check for the correct mutation.
- 20 ***IgG Quantification:***

Coat each well of a 96-well immunoplate with 100µl aliquots of 0.4µg/ml goat anti-human IgG antibody, diluted in PBS, incubate overnight at 4°C. (Plates may be stored for 1 month at this stage). Also coat another blank plate with BSA/PBS blocking solution. Remove the excess coating solutions and wash the plate three times with 200µl/well of washing buffer.
- 25

Into the blank plate, dispense 120µl of SEC buffer into all wells except the wells in column 2, rows B to G. Prepare a 1 µg/ml solution of the human IgG1/kappa antibody in SEC buffer to serve as a standard. Pipette 240µl/well into the wells in column 2, rows B and C. Centrifuge the medium from transfected cells (250g, 5mins) and save the supernatant. Pipette 240µl of the supernatant from the "no DNA" control (where cos cells were transfected in the absence
- 30

of DNA) into the well in column 2, row D. Pipette 240µl/well of experimental supernatants into the wells in column 2, rows E, F, and G. Mix the 240µl aliquots in the wells of column 2, rows B to G, and then transfer 120µl to the neighbouring wells in column 3. Continue to column 11

- with a series of 2-fold dilutions of the standard, control, and experimental samples. Transfer 100µl from each well to the corresponding well of the anti-IgG-coated plate. Incubate at 37°C for 1hr. Rinse all the wells 3x with washing buffer (200µl). Dilute the goat anti-human kappa light chain peroxidase conjugate 5000-fold in SEC buffer and add 100µl to each well. Repeat
- 5 the incubation and washing steps (step 9). Add 150µl of K-BLUE substrate to each well, incubate in the dark at RT for 10mins. Stop the reaction by adding 50µl of RED STOP solution to each well. Read the optical density at 655nm.

Lewis^y binding ELISA

- 10 Coat each well of a 96-well MaxiSorp plate (Nunc) with 100ng/well of Lewis Y HSA peptide in PBS and incubate overnight at 4°C. Wash 3x with PBS-T (0.1% Tween20). Block a fresh plate with 250µl of PBS/0.2% BSA/0.05% Tween20 per well and incubate for 1hr at RT. Wash 3x with PBS-T. Add 240µl of antibody (diluted in PBS/0.2% BSA/0.05% Tween20 if necessary) to wells in column 1; 120µl of buffer (PBS/0.2% BSA/0.05% Tween20) in the
- 15 other wells. Transfer 120µl from column 1 to the neighbouring wells in column 2. Continue to column 12 with a series of 2-fold dilutions of the experimental samples. Transfer 100µl per well from the dilution plate to the experimental plate. Incubate for 1hr at RT. Wash wells 3 x with PBS-T. Dilute the goat anti-human Fc peroxidase conjugate 10000-fold (or anti-mouse at 10000-fold dilution) in PBS/0.2% BSA/0.05% Tween20 and add 100µl to each well.
- 20 Incubate 1hr at RT and repeat washing step. Add 150µl of substrate (K-Blue) per well and incubate for 150mins at RT. Stop the reaction by adding 50µl of RED STOP solution to each well. Read the optical density at 650 nm.

Thermal stability

- 25 Dilute fully humanised antibodies and the chimeric control to 1µg/ml in TBS/0.2% Tween and aliquot at the appropriate volume for the EC80 concentration into PCR tubes. Bring the volume up to 100µl with the same buffer. Heat separately each tube for 10mins at temperatures between 30°C and 85°C with a 5°C interval and cool to 4°C. Freeze down the 1µg/ml stock for 1hr and then dilute to the EC80 concentration. Perform the binding assay
- 30 against Lewis Y HSA peptide using 100µl of each antibody per well (assay each temperature in duplicate) in a 96-well plate.

Thermal shift comparison

- Prepare samples directly into 96 well white PCR plate in a final volume of 25µl (purified
- 35 antibody final concentration of 1 and 2µM. Sypro Orange – make stock 1:100 in PBS buffer, then add 1:10 to final samples (e.g. 2.5µl in 25µl). Load into the qPCR machine and use the MxPro software, SYBR Green method, (filter = FRROX, no reference dye). Thermal profile setup – 71 cycles of 1° increase. Plot the results and determine T_m.

Purification of humanised mab

Instrument: GE Healthcare ÄKTAexpress™ Purification System

Software: UNICORN

Columns: HiTrap MabSelect SuRe, 1 ml; HiLoad 16/600 Superdex 200pg

5 Mobile phase: IgG Elution Buffer; Dulbecco's 1x PBS

Sample prep: Filtering through 0.22 µm

Injection volume: 200ml Expi293 conditioned medium (1:1) in DPBS

Flow rate: Sample loading at 0.5ml/min; Gel filtration at 1.5 ml/min;
Elution at 1ml/min

10

Aggregation comparison

Instruments: Agilent 1260 infinity HPLC system with thermostatted column compartment, Wyatt Technology Dawn Heleos, Wyatt Technology Optilab TReX

15 Analysis software: Wyatt Technology Astra version 6.1.1.17

Column: Acquity UPLC BEH200 SEC, 4.6 x 150mm, 1.7µm

Mobile phase: Dulbecco's 1x PBS with 0.05% sodium azide

Sample prep: Filtering through PES 0.22 µm

Sample concentration: Varying Injection volume: 20µl

20 Analysis temperature: 30°C

Flow rate: 0.4ml/min

Cross-interaction chromatography (CIC)

25 Cross-interaction chromatography (CIC) analysis was carried out to assess proneness to non-specific protein- protein interactions and provide an indication of any solubility issues, which can give rise to downstream manufacturing problems.

Samples were analyzed by two separate 20µl injections (0.5mg/ml); firstly onto a 1ml NHS activated resin (GE Healthcare) coupled with 30mg human polyclonal IgG (Sigma 14506) and secondly onto a 1ml NHS activated resin blank coupled, as control column. The mobile phase consisted of Dulbecco's PBS (Sigma D8537) containing 0.01% sodium azide (0.1ml/min) and all experiments were performed at 25°C. Eluted samples were detected by UV absorbance (Agilent 1260 Infinity HPLC system with thermostatted column compartment) and data was analysed using Wyatt Technology ASTRA software (version 6.1.2.83) to determine sample peak retention times. These were then used to calculate a retention factor k' :

$$k' = \frac{(Tr - Tm)}{Tm}$$

40

where T_r is the retention time of the sample on the poly-IgG column and T_m is the retention time on the mock (control) column.

Surface Plasmon Resonance (SPR): Lewis Y HSA conjugate was coupled to a CM5 chip and the FG27.10 and FG27.18 mAbs (concentration range 0.01 μ M-1 μ M) injected at 30 μ l/min. Binding data were fitted to a heterogenous ligand model using the BIA evaluation software.

Antibody internalisation: Cancer cells were plated at a density of 2x10³ cells/well and left to adhere overnight. Cells were cultured at 37°C for 72hrs in 96-well plates and treated with the indicated concentration of mAb in combination with secondary antibody: saporin-conjugated goat antibody (F'ab fragment) to mouse (ATSbio, IT#48) or human IgG. Control wells consisted of cells incubated with culture medium only and with secondary conjugate only. The amount of secondary antibody per well was 75ng and primary antibody concentration was varied from 0.01nM to 30nM. Saporin internalisation was assessed via inhibition of thymidine uptake (thymidine was added for the last 24hrs of the incubation).

ADCC and CDC: Cells (5x10³) were co-incubated with 100 μ l of PBMCs, 10% autologous serum or media alone or with mAbs at a range of concentrations. Spontaneous and maximum releases were evaluated by incubating the labeled cells with medium alone or with 10% Triton X-100, respectively. After 4hrs of incubation, 50 μ l of supernatant from each well was transferred to 96 well lumaplates. Plates were allow to dry overnight and counted on a Topcount NXT counter (Perkin Elmer, Cambridge, UK). The mean percentage lysis of target cells was calculated according to the following formula:

$$\text{Mean \% lysis} = 100 \times \frac{\text{mean experimental counts} - \text{mean spontaneous counts}}{\text{mean maximum counts} - \text{mean spontaneous counts}}$$

PI uptake assay: FG27.10 and FG27.18 were incubated with AGS cells and tested for uptake of the small molecular weight dye propidium iodide (PI, Sigma) at various concentrations; the number of PI positive cells representing the number of dying/dead cells. Tumour cells (5x10⁴) were incubated on a 96-well round bottom microtitre plate with 50 μ l of primary antibodies at RT for 2hrs. 1 μ g of PI was added and cells were incubated at RT for 30mins. Samples were analysed on a FC-500 flow cytometer (Beckman Coulter). To analyse and plot raw data WinMDI 2.9 software was used. For comparison, mAb SC104 which is known to induce membrane damage and 791T/36 were also included as internal controls for the experiment.

Proliferation inhibition assay: Proliferation inhibition by the constructs was assessed by using the water-soluble tetrazolium salt WST-8 (CKK8 kit, Sigma-Aldrich) to measure the activity of cellular hydrogenases which is directly proportional to the number of viable cells. Briefly, after

overnight plating of cancer cells (2000 cells/90µl/well), constructs were added at different concentrations in a final volume of 10µl/well and the plates were incubated at 37°C, (5%CO₂) for 72-96h. WST-8 reagent was then added (10µl/well) and after a further 3h incubation, the plates were read at 450nm (Tecan Infinite F50) and percentage inhibition calculated. EC₅₀ values were determined using nonlinear regression (curve fit) with GraphPad Prism v 8.0 (GraphPad Inc, La Jolla, CA).

In vivo model: The studies were conducted under a UK Home Office Licence. NCRI guidelines for the welfare and use of animals in cancer research, LASA good practice guidelines and FELAS working group on pain and distress guidelines was also followed. Endotoxin free (<10EU/ml) FG27 mAb was supplied in pre-formulated aliquots ready for dosing and stored at -20°C until use. Age matched male MF-1 nude mice were obtained from Harlan Laboratories (Bicester, UK) with each group, FG27, control mAbs or the vehicle control, consisting of n≥8 animals.

Mice were implanted with C170HM2 DLuX cells and monitored by optical imaging to determine tumour establishment and suitability to be entered into the study. Mice were dosed with either FG27 or a positive control mAb (505/4, FG88.2 or FG88.7 (1mg/ml) as appropriate) at 0.1mg 2 x weekly 100µl intravenously (i.v.) until termination, or PBS, the vehicle control for the mAb, 100µl 2 x weekly i.v. until termination. Weekly bioluminescent imaging was carried out on all mice to obtain pre and post dosing tumour measurements. In this way each mouse provided pre-dose control readings against which tumour growth could be compared.

All measurements and readouts were transferred from the original dictation/notation to excel (tab delineated) format for data processing in SPSS v16.0. Data integrity was checked using explore and descriptive functions. Erroneous points when identified were cross referenced against the original data and corrected accordingly. The data was screened for outliers and distribution profile; data-points falling outside the 95% confidence limit (outliers) were removed from analysis, but kept in the datasheet for reference purposes.

Mice were imaged weekly for bioluminescent tumour burden (BLI) over the duration of the study as follows; 60mg/kg D-Luciferin substrate was administered subcutaneously (s.c.), the mice were anaesthetised and BLI readings taken 15mins post substrate administration on open filter block (2D) and sequential emission filters (for DLIT, 3D reconstruction). Ventral and dorsal imaging was undertaken; the optimum position for imaging was abdomen uppermost. BLI was measured over the entire abdominal area, one Region of Interest (ROI) for each mouse in order to include all lesions present. Each mouse had a pre-dosing or baseline image taken to allow calculation of percentage tumour growth over time; these data were averaged per group. BLI readings were also taken after termination

to identify tumours in PM tissue.

EXAMPLE 1 – Generation and initial characterisation of FG27 mAbs:

FG27 was raised by immunisation with gastric tumour cell glycolipid

- 5 Analysis of antibody response to immunisations: Antibody titres were initially monitored by lipid enzyme-linked immunosorbent assay (ELISA). Thin layer chromatography (TLC) analysis using ST16 total and plasma membrane lipid extracts, flow cytometry analysis (FACS) using ST16 tumour cells and Western blot using ST16 whole cell extract, total and plasma membrane lipid extracts were subsequently performed. The mouse considered to
10 have the best response, compared to the pre-bleed serum control was boosted intravenously (i.v.) with ST16 plasma membrane lipid extract prior to fusion.

- Binding of FG27 hybridoma supernatant to a panel of tumour cell lines was analysed by direct immunofluorescence and FACS analysis. Both FG27.10 and FG27.18 bound ST16
15 but did not bind human umbilical vein endothelial cells (HUVECs) or peripheral blood mononuclear cells (PBMCs) when compared to positive control anti-HLA mAb, W6/32 (eBioscience, CA, USA), and the negative control (Figure 4). FG27.10 and FG27.18 were both cloned. FG27.10 was an IgG3k and FG27.18 was an IgG1k subclass. To ensure that both mAbs bound to glycolipid, glycolipid was extracted from a range of cell lines, dried
20 onto an ELISA plate before incubating with FG27.10 and FG27.18. Binding was seen with C170, ST16 and AGS derived glycolipid with both mAbs but not cell lines which showed a lack of binding to whole cells (MKN-45, OAW28, OVCAR-3, OVCAR-4 and OAW42; Figure 5).

25 EXAMPLE 2 – Defining the epitopes recognised by FG27 mAbs

- To determine the degree of specificity of FG27.10, the antibody was initially assessed for binding to the HSA-coupled Le^a, Le^b, Le^x and Le^y antigens by ELISA. FG27.10 failed to recognise Le^a, Le^{a-x} or Le^b but strongly recognised Le^y (Figure 6). To further clarify the fine specificities of the FG27 mAbs, they were screened against ≥600 natural and synthetic
30 glycans by the Consortium for Functional Glycomics. Binding of FG27.10 and FG27.18 mAbs to the glycan array showed that both mAbs bound specifically to Le^y (Figure 7). For comparison, binding of 692/29 demonstrated the mAb bound most strongly to Le^b, glycans containing Le^b and also to tri-Le^y and its variants. In contrast, BR96 was shown to bind Le^y as well as a range of Le^y variants (Le^{y-x}, Le^{y-x-x}) and more weakly to Le^x.

- 35 FG27.10 and FG27.18 were also screened on a panel of tumour cells lines by SDS/PAGE/Western blot for their ability to recognise glycoproteins as well as glycolipids (Figure 8). In addition to binding glycolipids, as evidenced by binding at the dye front, both mAbs, also recognised a range of glycoproteins.

40

As FG27 was Le^y specific, its binding to cell lines (Figure 9) was compared to mAb BR96, which recognises Le^y and Le^x, and mAb 692/29, which recognises Le^y and Le^b. All three mAbs bound C170 and AGS cells. In contrast, BR96 bound strongly to Colo201, 692/29 bound moderately whereas FG27 failed to bind (Figure 9). This shows that this cell line does not express Le^y but probably expresses Le^x and Le^b, which could not have been concluded using the non specific mAbs. HT29 cells failed to bind any of the mAbs whereas FG27.10 bound best to OVCAR3 and ST16 and 692/29 to LoVo cells.

EXAMPLE 3 – Immunohistochemistry assessment for FG27

To assess the binding of FG27 to normal human tissues it was screened on a panel of tissue types. In contrast to mAbs cross reacting with Le^{y/b} (692/29) and Le^{y/x} (BR96), FG27 only bound to subpopulations of cells within normal stomach, lung, tonsil, pancreas and duodenum (Table 1). Further, in contrast to other Le^y cross reactive mAbs, FG27 failed to stain colon, jejunum, breast, kidney or the ileum. Representative IHC images of the normal staining of the oesophagus, stomach, duodenum, ileum, colon, pancreas, lung and kidney are shown in Figure 10.

Table 1: Binding of FG27 to normal human tissues as assessed by immunohistochemistry (IHC). Staining of these tissue microarrays (TMAs) were analysed via new viewer software 2010 and given a semi-quantitative score according to intensity of staining of tumour tissue. Strong staining was given a score of 3, moderate staining a score of 2, weak staining a score of 1 and a negative score of 0. The results also demonstrate differential staining of specific cell types within these tissues.

Tissue type	692/29 (Le ^{y/b})	BR96 (Le ^{y/x})	FG27 (Le ^y)
Placenta	0	2	0
Oesophagus	0	3	0
Rectum	0	1	0
Gallbladder	1	2	0
Skin	0	0	0
Adipose	0	0	0
Heart	0	0	0
Skeletal	0	0	0
Bladder	0	0	0
Ileum	2	3	0
Spleen	0	0	0
Brain	0	0	0
Jejunum	3	3	0
Stomach	2	3	0/2
Breast	0	3	0
Kidney	0	1	0
Testis	0	0	0
Cerebellum	0	0	0
Liver	0	0	0
Thymus	1	0	0
Cervix	0	0	0
Lung	1	3	0/1
Smooth Muscle	0	0	0
Colon	1	1	0
Ovary	0	0	0
Tonsil	2	3	0/1
Diaphragm	0	0	0
Pancreas	1	3	0/2
Uterus	0	0	0
Duodenum	1	3	0/2
Thyroid	0	0	0

To determine the therapeutic value of FG27, it was screened on human gastric, colorectal, ovarian and breast TMAs by IHC. It stained 80% of gastric (69/86), 100% of colorectal (55/55), 95% of ovarian (36/38) and 85% of breast (39/46) tumour tissues stained at differing intensities (Table 2).

Table 2: Binding of FG27 mAb to human gastric, colorectal, ovarian and breast tumour tissues as assessed by immunohistochemistry. Staining of these tissue microarrays were analysed via new viewer software 2010 and given a semi-quantitative score according to intensity of staining of tumour tissue. Strong staining was given a score of 3, moderate staining a score of 2, weak staining a score of 1 and a negative score of 0.

Frequency and intensity of Lewis ^y expression on human tumour types					
Tumour type	No	Total positive	Immunohistochemistry (IHC) scores of Lewis ^y		
			3+	2+	1+
Gastric	86	69	19	25	25
Colorectal	55	55	29	18	8
Ovarian	38	36	2	18	16
Breast	46	39	7	13	19

Scoring showed that FG27 bound widely to gastric tumours with positive staining in 59% of tumours and 18% of tumours staining strongly. 19% of tumours stained moderately and 22% stained weakly. Results show that FG27 has the potential to target a wide range of gastric tumours and bind strongly to 18% of them. Kaplan Meier analysis revealed no association between expression of FG27 antigens and survival (data not shown). However, there was a correlation between FG27 antigen expression and M30 (apoptotic marker) expression (Pearson's chi-squared $p=0.049$). There was also a correlation between FG27 antigen expression and tumour site (Pearson's chi-squared $p=0.008$) with 65% of stomach specific tumours staining with FG27 with 26% staining strongly. In contrast, only 53% of gastro-oesophageal joining regions tumours stained but 37% of these stained strongly. 46% of tumours in the lower one third of the oesophagus stained with FG27 with 53% of them staining strongly.

Due to cross-reactivity of FG27 with ovarian cancer cell lines, it was screened against an ovarian cancer TMA with 360 tumours by immunohistochemistry. Scoring showed that FG27.18 bound poorly to ovarian tumours with 85% of tumours staining negatively. Only 15% of tumours stained positively and only 3% stained strongly. 3% of tumours stained moderately and 9% stained weakly.

Kaplan Meier survival analysis showed that patients whose tumours expressed FG27 antigen had a significantly (log rank $p=0.049$) better survival than those with no expression. There was a correlation between FG27 antigen expression and FIGO stage (Pearson's chi-squared $p=0.024$). There was also correlation between FG27 antigen expression

- and types of ovarian cancer (Pearson's chi-squared $p=0.024$) with over a third of mucinous and endometrioid tumours expressing FG27 whereas less than 14% of serous, clear cells and undifferentiated tumours stained with this mAb. Cox multivariate analysis showed that FG27 did not confer any further prognostic value over FIGO stage, residual disease and response to chemotherapy.

Table 3. Comparison of staining tumours with the Lewis y specific mab FG27 with other Lewisy cross reactive mabs BR96 (Lewisy/x) and 692/29 (Lewisy/b).

A)					
FG27.10 VS 692/29 on human tumour microarrays					
Tumour type	No.	Both positive	Both negative	FG27.10 only	692/29 only
Breast	41	21 (51%)	4 (10%)	16 (39%)	0 (0%)
Ovarian	38	21 (55%)	1 (3%)	15 (39%)	1 (3%)
Gastric	72	59 (82%)	3 (4%)	2 (3%)	8 (11%)
Colorectal	52	43 (83%)	0 (0%)	9 (17%)	0 (0%)
b)					
FG27.10 VS BR96 on human tumour microarrays					
Tumour type	No.	Both positive	Both negative	FG27.10 only	BR96 only
Breast	40	19 (48%)	3 (8%)	17 (43%)	1 (3%)
Ovarian	40	19 (48%)	2 (5%)	19 (48%)	0 (0%)

Gastric	62	43 (69%)	6 (10%)	4 (6%)	9 (15%)
Colorectal	51	38 (75%)	0 (0%)	13 (25%)	0 (0%)

The monospecific Lewis y mab, FG27.10, stained 3-39% more tumours than the Lewis y/b 692/29 mab and 6-48% more tumours than the Lewis y/x Br96 mab, reflecting its unique specificity. The greatest variation was seen on the ovarian and breast tumours.

5

Unlike FG27, which only demonstrated moderate binding to normal stomach, pancreas and duodenum in one of two samples, and weak binding to lung and tonsil in one of two samples, BR96 demonstrated strong binding to a number of tissues including oesophagus, ileum, jejunum, stomach, breast, lung, tonsil, pancreas and duodenum. BR96 also demonstrated moderate binding to the placenta and gallbladder, and weak binding to the rectum, kidney and colon. In comparison, 692/29 bound the jejunum strongly, the ileum, stomach and tonsil moderately, and the gallbladder, thymus, lung, colon, pancreas and duodenum weakly (Table 1). This limited normal binding profile of FG27, particularly in regard to the gastro-intestinal tissues, did not diminish the binding of the antibody to tumour tissues with 80% of gastric and 100% of colorectal tumour tissues demonstrating antigen expression. In comparison, 692/29 and BR96 displayed a good distribution on colorectal tumours binding 82% and 90% respectively (Noble, Spendlove et al. 2013), a similar figure for the later, 15/18 samples i.e. 83% also being reported in US 5491088. 39% and 49% of these tumours stained moderately or strongly compared to 85% for FG27. 2-25 LE showed a poorer distribution, binding only 52% of colorectal tumours with 19% staining moderately and none staining strongly (Noble, Spendlove et al. 2013).

It is therefore demonstrated the FG27 has a significantly better normal binding profile, particularly in regard to the gastro-intestinal tissues, and the differential binding between each of Le^y mAbs indicates the distinct nature of the invention.

EXAMPLE 4 – *In vivo* anti-tumour activity of FG27 in an established colorectal metastatic xenograft model

Comparison of the therapeutic effect of the mAb FG27 in the C170HM2 DLUx human hepatic metastasis model: The mouse C170HM2 DLUx human hepatic metastasis tumour model was used to investigate the anti-tumour activity of the murine FG27 mAb. The C170HM2 DLUx cell line is a bioluminescent variant of a liver metastasising colon tumour cell line passaged to invade the liver parenchyma when implanted into the

peritoneal cavity and left 10 days to metastasise to the live before mAb treatment was started. Growth and distribution/location of such labelled cells and tissue can be assessed non-invasively in real time and in excised tissue at post mortem (PM) in a suitable optical imaging system.

5

Anti-tumour data: FG27 administered twice a weekly (100µg i.v.) reduced peritoneal cavity and associated tumour growth compared to the vehicle control as assessed by bioluminescent intensity (Figure 11a). The study was terminated on day 32. Treatment with FG27 resulted in a significant reduction in bioluminescent tumour burden by the last day of the study compared to the vehicle control ($p=0.014$). There was a significant reduction in injection site tumour growth with FG27 treatment ($p=0.05$; Figure 11b).

In a second *in vivo* study extending the duration of treatment, FG27 demonstrated a significant reduction in percentage tumour growth by day 51 ($p=0.019$) compared to the treatment control group (Figure 12a). Analysis by Log Rank Mantel-Cox test demonstrates significant enhanced survival in the FG27 ($p=0.0251$) treatment group compared to the vehicle only control (Figure 12b).

EXAMPLE 5 – Chimeric mAb

The term “chimeric antibody” is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody. Chimeric (or humanised) antibodies of the present invention can be prepared based on the sequence of a murine mAb prepared as described above. When the gene and amino acid sequence of the antibodies was analysed it showed that FG27.10 (Figure 1) and FG27.18 (Figure 2) had identical variable regions but different constant regions IgG3 and IgG1 respectively. This suggested that a single B cell had subclass switched and then expanded during the immunisation protocol and that both of these clones were successfully fused.

Compared to the closest germline sequences, FG27 contains 7 mutations in its heavy chain (all non-silent) and 3 mutations in its light chain (all non-silent). 4 amino acid differences to the germline are in the heavy chain CDRs and 3 in FR3. In the CDR1, there is an amino acid change from D to H at position 36. In the CDR2, the amino acid changes are a G to D at positions 59 and 63 and at position 64, S to N (Figure 2a). For the light chain, there are 2 amino acid differences in the CDR1 germline, S to I at position 28 and S to T at position 32, and 1 in the FR2, K51N which is unique.

There are several other mabs that bind Lewisy but cross react with other related Lewis antigens. FG27.10/18 are the only antibodies with monospecificity for just Lewis y. This is reflected in the similar but distinct sequence of the FG27 variable heavy and light chain regions (figure 13a,b). FG27 shows 16-68% differences in the amino acid sequences CDRs within the heavy chain, 4.3 – 16% differences in amino acid differences in the CDR sequence in the light chain, 8.6-49.5% differences in amino acid sequence in the variable region of the heavy chain, 2-24% differences in amino acid sequence in the variable region of the light chain from the other cross reactive lewis y mabs (Table 4). The most variation is within CDRH2 with 3-5 of the 8 amino acids varying between the different mabs.

10

Table 4. Differences in amino acid sequences in the variable heavy and light regions of FG27 from anti-lewis y cross reactive mabs.

mab	FR1	CDRH1	FR2	CDRH2	FR3	CDRH3	TOTAL	TOTAL CDRs Diff fg27	TOTAL Diff fg27
	25	8	17	8	38	9	105	25	
FG27	0	1	0	3	3	ARGKYDGAW	6.7	-	
S56976	0	0	1	3	0	same	3.8	16	8.6
H18A	3	0	1	3	1	same	7.6	16	11
BR96	1	0	0	3	0	2	2.9	20	8.6
PASTAN B3	1	0	3	4	5	4	12.4	32	11.4
HU38193	1	0	3	5	10	4	18.1	32	19
SC101	13	5	8	3	23	9	49.5	68	49.5
mab	FR1	CDRL1	FR2	CDRL2	FR3	CDRL3	TOTAL	TOTAL CDRs Diff fg27	TOTAL Diff fg27
	26	11	17	3	36	9	102	23	
FG27	0	0	2	1	0	0	3	-	
S56976									

H18A	1	1	2	0	0	0	4	8.7	3
BR96	2	2	1	0	0	0	5	4.3	4
PASTAN B3	0	1	0	0	0	0	1	4.3	2
HU38193	9	1	4	0	10	0	24	8.7	24
SC101	1	0	0	1	0	0	2	16	6

FG27.18 heavy and light chain variable regions were cloned into human IgG1 and IgG2 expression vectors. This was transfected into CHO-S cells and human antibody purified on protein G. The chimeric mAbs bound to Le^y expressing tumour cell lines, including the gastric cell line, AGS (Figure 14).

EXAMPLE 6 – Direct cell killing

A number of anti-glycan mAbs have been shown to induce direct cell death in antigen positive cell lines with no need for effector cells or complement. This can potentially enhance their *in vivo* efficacy as tumours can develop mechanisms to avoid immune-mediated cell death. This capability is mainly associated with glycan-binding mAbs of the mIgG3 isotype, hence the property is lost upon chimerisation or humanisation.

In order to determine whether FG27.10 and FG27.18 have the ability to cause direct cell death, ST16 cells were incubated with FG27.10, FG27.18 and the anti-sialyl Le^a mAb SC104 for 2hrs at RT, before cell death was measured by the uptake of propidium iodide, which is a DNA intercalating agent that is only taken up by dying cells (Figure 15a). Interestingly, despite having the same variable region, only FG27.10 was able to induce direct cell death, with no uptake of PI observed with FG27.18. Figure 15b shows direct killing of a range of antigen positive (AGS, Colo201, C170, C170HM, MCF-7, OVCAR4) cell lines with the two mAbs showing variable amounts of killing. Neither mAb showed killing of the antigen negative cell lines (LoVo, MKN45, 791T, SKOV3).

Figure 15c shows PI uptake in the colorectal cell line C170 by a range of mAbs. FG27.10 and the positive control antibody SC101 showed strong titratable killing. FG27.18 showed weak non titratable killing. The human IgG1 chimeric showed moderate titratable killing whereas the IgG2 variant did not.

EXAMPLE 6 – Internalisation

FG27.10, FG27.18 and CH27 were all assessed for internalisation using a variety of tumour cell lines (Figure 16). FG27.18 demonstrated dose-dependent internalisation on most cell lines tested with decreases in cell viability ranging from 60% (C170) up to 75%

(MCF7) and 85% (AGS) at the highest concentration tested. FG27.10 showed best internalisation on AGS (up to 50% decrease in viability) and MCF7 (up to 70% decrease in viability). Internalisation on the H322 cell line was more variable for both the murine mAbs. The CH27 mAb was efficiently internalised by MCF7, with up to a 75% reduction in cell viability being observed, and by H322 with a ~40% reduction in cell viability.

EXAMPLE 7 – *In vitro* anti-tumour activity of FG27

ADCC and CDC: The ability of murine and chimeric FG27 mAbs to induce tumour cell death through ADCC was screened. Human PBMCs were used as the source of effector cells while a panel of tumour cells served as target cells. Initially ADCC analysis was undertaken with FG27.10 IgG3 supernatant (Figure 17a). The extent of ADCC seen corresponded to expression of the Lewis Y antigen and was therefore seen for ST16 and OVCAR3 with only low levels being observed with C170s and background alone for LoVo, HT29 or Colo201 (Figure 17a). The extent of ADCC MCF7 and OVCAR3 target cell killing by murine FG27.10 (mIgG3), FG27.18 (mIgG1), CH27 IgG1 and IgG2 was measured and compared after 18hrs incubation at 37°C; the CH27 IgG1 gave the highest level of killing of the cell lines, MCF7 and OVCAR3 (Figure 17b).

CDC is known to be an important mechanism involved in eliminating tumour cells *in vivo*. The capacity of ST16 cells to be killed by CDC induced by the mAbs FG27.10, FG27.18, CH27 IgG1 and CH27 IgG2 in the presence or absence of human serum as source of complement at 37°C for 18hrs was assayed. As shown in Figure 17c, of the FG27 mAbs, FG27.10 (mIgG3) gave the highest level of killing at 3µg/ml whereas no significant levels were seen for the CH27 IgG2 which, given the nature of IgG2 antibodies, was as expected. Likewise, FG27.10 gave the highest level of killing, followed by the CH27 IgG1 when AGS cells were used. Again no killing was seen with the CH27 IgG2 (Figure 17d).

In summary, the FG27 mAbs strongly induced ADCC using human PBMCs as effector cells as well as significant CDC with human serum as a complement source.

EXAMPLE 9 – *Generation of a humanised version of the FG27 antibody*

Human VH and VK cDNA databases

The protein sequences of human and mouse immunoglobulins from the International Immunogenetics Database 2009 (Lefranc 307-10) and the Kabat Database Release 5 of Sequences of Proteins of Immunological interest (last update 17-Nov-1999)(Kabat et al. 1-3242) were used to compile a database of human immunoglobulin sequences in Kabat alignment. Our database contains 10,906 VH and 2,912 VK sequences.

FG27 Human framework selection

Humanisation requires the identification of suitable human V regions. The sequence analysis

program, Gibbs, was used to interrogate the human VH and VK databases with FG27 VH and VK protein sequences using various selection criteria. Using the program Discovery Studio (Accelrys), FW residues within 4Å of the CDR residues (Kabat definition) in the structures of mouse FG27 antibody were identified, and designated as the “4Å Proximity Residues”. Human VH sequences with highest identity to FG27 VH in the 4Å Proximity Residues are shown in Figure 19, together with a list of these envelope residues and VCIs, and the number of residues in either the FW, VCI or 4Å Proximity Residues which are identical to the mouse equivalent position. Figure 18 shows the alignment and residue identity of all the VH sequences in Figure 19. The number of apparent somatic mutations in each FW, identified by comparison with the most identical human germline V gene, is indicated in Figure 20.

Humanised sequences, murine and incomplete sequences were removed from the analysis. The sequence AJ579110 was chosen as the human donor candidate due to its higher sequence identity and similarity. It has no somatic mutations from its germline ABO19439 or HM855436 .

Design of FG27 RHA and FG27 RHB

As a suitable human framework has been identified, the synthetic protein and DNA sequence can be designed. The initial design of the humanised version of FG27 is the grafting of CDR 1, 2 and 3 from FG27 VH into the acceptor FW of AJ579110 . One VCI + 4Å Proximity Residue, at Kabat position 24 is not conserved in FG27 RHA, and this is back-mutated to the mouse equivalent residue to produce the humanised version FG27 RHB. Figure 21 compares the murine and the humanised versions of FG27 VH protein sequences.

FG27 RKA framework selection

In order to humanise the light chain a human kappa chain was identified in a similar process to that of the heavy chain. Figure 22,23 show 4Å Proximity Residues and the number of residues, in the FW or the 4Å Proximity Residues, which are identical to the mouse equivalent position, alignment of VK sequences and comparison to human germline. Initial analysis found several potential donor candidates, but all these proved to be Human VK4 which show poor expression. Extending the analysis to include CDR1 with one less residue resulted in a single candidate, X72449, which showed a higher degree of sequence homology to the murine antibody than the VK4 candidates. This candidate has a single somatic mutation from the human VK2 germline, but no somatic mutations from the human VK2D germline (figure 24).

Design of FG27 RKA and FG27 RKB

The framework from X72449 was used to design the DNA and protein for the humanised constructs. CDR 1, 2 and 3 from FG27 VK are shown grafted into the acceptor FW of

X72449 to generate the initial version of humanised FG27. There is a single unmatched 4Å Proximity residue, 5, in FG27 RKA that was back-mutated to the equivalent mouse residue in variant FG27 RKB (Figure 25).

5 **Generation and properties of a humanised version of FG27**

Generation of FG27 humanised antibodies

The genes for FG27 RHA and FG27 RKC were synthesized by Genewiz and codon optimised for human sequences (Figure 3ab). The natural human framework sequences M65092 and X72449, heavy and light chains, respectively, and the natural mouse CDR sequences were assembled *in silico* and designated FG27 RHA to FG27 RHB and FG27 RKA to FG27 RKB. Using software algorithms proprietary to Genewiz, the sequences were optimised by silent mutagenesis to use codons preferentially utilised by human cells and synthesized. RHA/B and RKA/B constructs were PCR amplified with specific primers to the expression vector + insert (as described previously for the chimeric versions) and inserted into pHuG1 and pHuK in ligase independent cloning reactions and used to transform TOP10 bacteria. RKA and RHA were subsequently modified by PCR mutagenesis to obtain all human variants annotated in the Figure 21 and 25.

Clones were sequenced and expression plasmid DNA was prepared using the QIAGEN Plasmid Miniprep Kit or Qiagen Plasmid Maxiprep kit. The expression construct sequences (RHA, RHB, RKA and RKB) are shown in Figure 21 and 25. Expression plasmid preparations encoding (humanised or chimeric) VH and VK were used to transfect Expi293 cells, cultured for 10 days in serum free media, whereupon the conditioned medium containing secreted antibody was harvested.

Antibody expression

The concentrations of IgG1k antibodies in Expi293 cell conditioned media were measured by ELISA and are shown in Table 5. Most antibodies were produced at a satisfactory level, except for those highlighted in red.

Table 5. IgG levels in transfected Expi293 cell conditioned medium

CONSTRUCTS USED		IgG CONCENTRATION (ng/mL) IN
VK	VH	µg/mL
1210VH	1210VK ctrl	256.39
FG27	FG27	20.19
FG27	FG27	112.34

LewisY-HSA binding by FG27 antibodies

Binding activity to the LewisY-HSA antigen was measured by Binding ELISA. Initial experiments showed there was essentially no difference between the binding potency of the mouse or chimeric antibodies. This result gives us some confidence in the success of the

humanisation process.

The data shown in Figure 26 show the binding potency of the initial versions of the humanised FG27. Although all versions bound to LewisY-HSA. Versions of the antibody
5 consisting of RHA/RKB, RHA/RKC, RHA/RKD and RHB/RKC were chosen as possible lead candidates.

Thermo stability of humanised candidate antibodies to high temperatures

The aim of this experiment is to compare the thermo stability of the humanised antibodies.
10 When subjected to higher temperatures, varying from 30° to 85°C for 10mins, cooled to 4°C and used in an ELISA assay at the EC80 concentration of each candidate. All the antibodies tested appear equally stable (Figure 27), all becoming inactive at >72°C.

Determination of humanised candidate antibodies T_m

15 In order to determine the melting temperature of the chimeric and humanised lead antibodies RHA/RKB, RHA/RKC, RHA/RKD and RHB/RKC all antibodies were purified in a 2-step affinity chromatography and gel filtration system and tested in a thermal shift assay. Samples were incubated with a fluorescent dye (Sypro Orange) for 71 cycles with 1°C increase per cycle in a qPCR thermal cycler. T_m for the chimeric and the four humanised antibodies are
20 indicated in Figure 28. All of the candidate antibodies and the chimeric antibody have the same T_m's (72°C).

Affinity of humanised candidate antibodies

Antibody affinity determination using SPR analysis was carried out using a Biacore T3000.
25 Lewis Y-HSA was covalently immobilised to a CM5 chip (Cat: BR-1005-30) in accordance with the manufacturer's instructions. Chimeric FG27, RHA/RKB, RHA/RKC, RHA/RKD and RHB/RKC antibodies were diluted in buffer in concentrations ranging from 200nM to 0.274nM (serial 3-fold dilutions) and then injected at a flow rate of 50µl/min. Association time was set at 120secs and disassociation time 600secs.

30 The kinetics of binding/dissociation was analysed according to the best fit to heterogenous ligand model using BIAcore 3000 evaluation software package. An overlay of the 200nM binding curves is shown in the sensogram (Figure 29). RHA/RKB, RHA/RKC, and RHB/RKC show similar affinities to chimeric FG27 (RHA/RKB>RHA/RKC>RHB/RKC >> RHA/RKD.
35 These results suggest that the humanisation has successfully retained the binding activity within the desired parameters and RHA/RKB or RHA/RKC have the highest affinities.

Aggregation of humanised candidate antibodies

Samples were injected at 0.4ml/min into a size exclusion column in an HPLC system and
40 analyzed by multi- angle light scattering to determine the absolute molar masses and check

for aggregation. All variants show no signs of aggregation with an average molecular weight ranging of 140.19 kDa, which is the expected range for an IgG monomer in this analysis setup. All samples are monodispersed ($M_w/M_n < 1.05$). The mass recoveries (calculated mass over injected mass) are 100% which indicates good protein recovery and that the samples do not seem to stick to the column or contain insoluble aggregates, which would be retained by the guard column. Overall the data suggest there are no aggregation concerns in any of the anti-FG27 antibody samples analysed.

Non-specific Protein-Protein Interactions (CIC)

Cross-Interaction Chromatography using bulk purified human polyclonal IgG is a technique for monitoring non-specific protein-protein interactions, and can be used to discriminate between soluble and insoluble antibodies. An elevated Retention Index (k') indicates a self-interaction propensity and a low solubility. RHA/RKB, RHA/RKC, RHB/RKC antibodies shows a Retention Index below 0.044, indicating a low propensity for non-specific interactions and good solubility (Figure 30).

The antibody has been engineered and expressed as a fully humanised antibody without losing Lewis Y HSA binding potency (Figures 26 and 29). Experiments with chimeric antibodies, consisting of murine variable regions on human constant regions, showed a similar potency in a Lewis Y HSA binding ELISA to that of the murine antibody. This result lends some confidence in a positive outcome for the humanisation process.

The initial experiments showed that the fully humanised heavy and light chains, i.e. without framework mutations to introduce murine 4A proximity residues, does not bind the Lewis Y HSA peptide as well as the chimeric positive control antibody but the versions with a single back mutation bind on a par with the chimeric positive control. Assays measuring antibody solubility, thermostability and aggregation suggest all the humanised candidates have acceptable biophysical characteristics.

In our view the combination of the excellent binding, expression, thermostability, affinity and cell binding makes RHA heavy chain version, in conjunction with the RKC light chain version the best candidate antibody for further development.

Example 10 – FG27 binding studies

The humanised (humanized 27) and chimeric mAbs (CH27) demonstrated a similar cell binding pattern on titration on cancer cell lines (Figure 31). Equilibrium affinity constants are overall on the same order, but slightly higher compared to the parental murine antibodies, whereas the maximum binding capacity of the humanised 27 and CH27 are larger compared to those of the murine mAbs. This result suggests a successful outcome of the humanization process.

Example 11 – Direct killing of the humanised mab

In order to enhance the direct cell killing of the human IgG1 27 chimeric (27hlgG1), we transferred selected mlgG3 constant region residues into the hlgG1 Fc domain thereby creating an improved
 5 'i27G1' Lewis^y glycan binding mAb with improved *in vitro* direct cell killing ability. Figure 33 shows the amino acid and nucleic acid sequences of the respective light and heavy chains of the 'i27G1' antibody. This antibody forms another aspect of the invention.

Figure 32 shows direct cell killing, functional affinity as well as effector functions of the improved
 10 i27G1. The mlgG3 27 antibody has direct cell killing ability on the Lewis^y-expressing AGS (i) and MCF7 cell lines (ii) whereas the chimeric 27hlgG1 does not. Our i27G1, containing selected mlgG3 constant region residues, displays significantly improved direct cell killing, compared to 27hlgG1, equivalent to the 27mG3 mAb, on both AGS (i) and MCF7 (ii). Additionally, i27G1 exhibits significantly improved functional Lewis^y affinity compared to the 27hlgG1 (iii). Importantly,
 15 the effector functions of i27G1, ADCC as well as CDC, were both equivalent to those of 27hlgG1, on MCF7 (iv and v). Collectively the results indicate that transfer of selected regions from the mlgG3 constant region into the 27hlgG1 backbone created a hybrid mAb with direct cell killing ability, increased functional affinity as well as robust immune effector functions.

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Claims

1. An isolated specific binding member capable of binding specifically to Fuca1-
5 2Galb1-4(Fuca1-3)GlcNAc (Le^x).
2. A binding member as claimed in claim 1, comprising one or more binding domains selected from the binding domains having the amino acid sequence substantially as set out as residues 27 to 38 (CDRH1), 56 to 65 (CDRH2) or 105 to 116 (CDRH3) of Figure
10 1a or 1b.
3. A binding member as claimed in claim 2, comprising the amino acid sequence substantially as set out as residues 1 to 127 (VH) of Figure 1a or 1b.
- 15 4. A binding member as claimed in any preceding claim, comprising one or more binding domains selected from the binding domains having the amino acid sequence substantially as set out as residues 27 to 38 (CDRL1), 56 to 65 (CDRL2) or 105-113 (CDRL3) of Figure 1c.
- 20 5. A binding member as claimed in claim 4, comprising the amino acid sequence substantially as set out as residues 1 to 124 (VL) of Figure 1c.
6. A binding member as claimed in any preceding claim, comprising residues 1 to 127 (VH) of the amino acid sequence of Figure 1a or 1b, and residues 1 to 124 (VL) of the
25 amino acid sequence of Figure 1c.
7. A binding member as claimed in claim 2 or claim 4, wherein the or each binding domain is carried by a human antibody framework.
- 30 8. A binding member as claimed in any preceding claim, further comprising a human constant region.
9. A binding member as claimed in any preceding claim, wherein the binding member is an antibody or an antibody fragment.
35
10. A binding member as claimed in claim 8 or claim 9, comprising a heavy chain amino acid sequence substantially as set out in Figure 2a and a light chain amino acid sequence substantially as set out in Figure 2b.
- 40 11. A binding member as claimed in claim 1, 2 or 4, comprising a heavy chain amino

acid sequence substantially as set out in Figure 3a and a light chain amino acid sequence substantially as set out in Figure 3b.

12. An isolated specific binding member capable of binding specifically to Fuca1-
5 2Galb1-4(Fuca1-3)GlcNAc (Le^x), which competes with an isolated specific binding member as claimed in any one of claims 2 to 11.
13. A binding member as claimed in any preceding claim, which is attached or
10 otherwise associated with a chemotherapeutic or cytotoxic agent.
14. A pharmaceutical composition comprising a binding member as claimed in any
preceding claim and a pharmaceutically acceptable excipient, diluent, carrier, buffer or
stabiliser.
- 15 15. A binding member as claimed in any one of claims 1 to 12 for use in medicine.
16. A binding member as claimed in any one of claims 1 to 12 for use in a method of
treating or preventing a tumour.
- 20 17. Products containing a specific binding member of any one of claims 1 to 12 and
an active agent, as a combined preparation for simultaneous, separate or sequential use in
the treatment of a tumour.
18. A binding member for use as claimed in claim 14 or claim 15 or products as
25 claimed in claim 16, wherein the tumour is a colorectal, gastric, pancreatic, lung, ovarian or
breast tumour.
19. A nucleic acid encoding a binding member as claimed in any one of claims 1 to
12.
30
20. A binding member comprising a heavy chain having an amino acid sequence
substantially as set out in Figure 33 and/or a light chain having an amino acid sequence
substantially as set out in Figure 33.
- 35 21. The binding member as claimed in claim 20 comprising a heavy chain encoded
by a nucleic acid sequence substantially as set out in Figure 33 and/or a light chain encoded
by a nucleic acid sequence substantially as set out in Figure 33.