

Modified Fc-regions to enhance functional affinity of antibodies and antigen binding fragments thereof

The present invention relates to the identification of key residues within mouse IgG3 antibodies (mAbs) that are responsible for intermolecular cooperativity and their transfer into IgG1 antibodies in order to enhance their functional affinity and direct cell killing.

Background

Mouse (m) IgG3 is highly protective against bacterial infection and is the only isotype among the mIgGs that forms non-covalent oligomers, strongly influencing their biological activity (Abdelmoula et al. 1989), and increasing functional affinity to polyvalent antigens. The tendency of mIgG3 oligomerisation was initially identified by Grey et al. (Grey, Hirst, and Cohn 1971) who showed that binding to multivalent antigens promoted mIgG3 intermolecular interactions, which resulted in increased functional affinity to antigen (Greenspan, Monafó, and Davie 1987; Greenspan and Cooper 1993), a characteristic termed 'intermolecular cooperativity' (Greenspan and Cooper 1993; Greenspan, Dacek, and Cooper 1989; Greenspan and Cooper 1992). It was determined the phenomenon depended on Fc as IgG3 F(ab')₂ fragments did not bind to the antigen cooperatively (Greenspan, Monafó, and Davie 1987). The mIgG3 isotype is associated with a propensity for intermolecular cooperativity on repetitive antigen, whereby the low monovalent affinity is rescued by avidity-mediated enhanced binding (Greenspan and Cooper 1993, 1992). Non-covalent interactions between adjacent mIgG3 Fc regions are thought to underpin this increased functional affinity, via prolonging target occupancy and reducing dissociation rates (Cooper et al. 1991; Greenspan, Dacek, and Cooper 1989).

The tumour glycome is a rich and under-explored source of cancer-specific targets for mAb development due to the alterations associated with the transformation process (Pinho and Reis 2015). Glycan structures are present on both protein and glycolipid backbones and can be massively over-expressed in cancer due to the altered expression of glycotransferases. Glycans have been shown to be key co-accessory molecules for cancer cell survival, proliferation, dissemination and immune evasion; crosslinking these antigens can lead to direct cell death without the need for immune effector cells or complement (Dalziel et al. 2014; Rodríguez, Schetters, and van Kooyk 2018; Rabu et al. 2012). Indeed, the inventors have generated a number of glycan-targeting mAbs with potential application for cancer therapeutics due to their strong differential tumour versus healthy tissue binding, high functional affinity and their ability to cure mice of metastatic colorectal cancer (Chua et al. 2015; Durrant et al. 2006; Noble et al. 2013), a number of other mAbs are in clinical development as passive or active immunotherapy, or reformatted for chimeric antigen receptor (CAR) T cells (Burris et al. 2010; Labrada et al. 2018; Hege et al. 2017) whilst Dinutuximab beta, an anti-GD2 mAb, is currently used for the treatment of neuroblastoma (Ladenstein et al. 2018).

The inventors have previously described a panel of cancer glycan targeting mAbs with Lewis^{a/c/x}, Lewis^y, as well as sialyl-di-Lewis^a reactivity (WO2017/033020A1; WO2015/063500A1; Chua et al. 2015; Noble et al. 2013). Intriguingly, if the glycan-binding mAbs were of the mIgG3 isotype they exhibit a direct cytotoxic effect on high-density target expressing cancer cells, independent of the presence of complement or immune effector cells, with the potential to initiate an adaptive anti-tumour immune response and long-term tumour control. This suggested that the intermolecular co-cooperativity enabled by the mIgG3 isotype was responsible for the direct cell killing. This direct cytotoxic ability has also been observed for other anti-glycan mAbs and typically involves mAb-induced homotypic cellular adhesion, cytoskeletal rearrangement followed by cell swelling, membrane lesions and eventual cellular demise (Loo et al. 2007; Faraj et al. 2017; Roque-Navarro et al. 2008; Chua et al. 2015; Welt et al. 1987). In most cases the cell death is a form of non-classical apoptosis, potentially involving the generation of reactive oxygen species (ROS) generation, most closely resembling oncotic necrosis (Zheng et al. 2017; Hernandez et al. 2011). Importantly, akin to immunogenic or inflammatory cell death (ICD), the coinciding release of inflammatory mediators - damage associated molecular patterns (DAMPs) - has the potential to recruit innate immune cells to the tumour site that may further increase mAb-mediated effector functions (Galluzzi et al. 2017). Thus, these anti-glycan mAbs can be important tools to remobilise the full potential of the immune system in an otherwise immunosuppressive environment.

For human antibody therapeutics, the areas not responsible for target specificity are typically replaced with their human equivalent, to generate a humanised version of the antibody which is clinically useful. When we chimerised our mouse mAbs to IgG1 (e.g. human IgG1, hIgG1) they lost direct cell killing and had reduced functional affinity as intermolecular cooperativity is not present in human IgGs. It has previously been suggested that mouse IgG3 antibodies might combine together once bound to their target, in a way other antibody subclasses do not, to stabilise binding and amplify the clinical potency of the antibody.

US2011/0123440 describes altered antibody Fc-regions and the uses thereof. The altered Fc-regions have one or more amino acid substitutions. This patent claims any substitution at many sites but the exact examples are all different substitutions to our invention exemplifying these substitutions are not responsible for direct cell killing.

US2008/0089892 describes polypeptide Fc-region variants and compositions comprising these Fc-region variants with amino acid substitutions in positions 251, 256, 268, 280, 332, 378 and 440 to improve ADCC. The only similarity with the modifications outlined in this patent was position 378 but we substitute it with a serine rather than an aspartic acid and we show decreased ADCC.

US2010/0184959 describes methods of providing an Fc polypeptide variant with 2 or more amino acid substitutions and altered recognition of an Fc ligand (e.g. FcγR or C1q) and/or effector function (e.g. ADCC). In contrast our substitutions are only related to increased cell killing. The

presently disclosed 26 and 23 amino acid motifs do include V305A, Q342R R344Q substitutions whereas the 15 amino acid motif only contains Q342R.

5 US2010/015133 describes methods of producing polypeptides by regulating polypeptide association. They claim changing the charge of paired amino acids from negative to positive charge or the reverse in the CH3 constant region of an antibody at position 356/439, 357/370 and 399/409. In contrast this patent described changing amino acid 356 from aspartic acid (D) to glutamic acid (E) which does not change charge and amino acid 357 from Glutamic acid (E) to Glutamine (Q) which changes a negative charge to neutral and amino acid 370 from lysine (K) to
10 threonine which changes a negative charge to neutral.

WO2018/146317 describes polypeptides and antibodies having an Fc region and an antigen-binding region where the Fc region has an Fc-Fc enhancing mutation and a C1q binding-enhancing mutation providing for polypeptide or antibodies with increased CDC activity
15 and/or agonistic activity. The Fc region is said to comprise (a) a substitution at a position selected from the group consisting of: E430, E345 or a S440Y or S440W substitution, and (b) a substitution at one or more position(s) selected from the group consisting of: G236, S239, S267, H268, S324, K326, I332, E333 and P396 wherein the positions correspond to human IgG1. The polypeptide comprises at least one substitution from the group consisting of: E430G, E345K, E430S, E430F,
20 E430T, E345Q, E345R, E345Y, S440Y and S440W. The present invention does not include any of these substitutions.

WO2018/083126 describes polypeptides and antibodies comprising a variant Fc region. The Fc region provides for stabilised Fc:Fc interactions when the polypeptide(s), antibody or antibodies are
25 bound to its target, antigen or antigens on the surface of a cell, while at the same time also having decreased complement-dependent cytotoxicity (CDC) and may also have decreased activation of other effector functions resulting from one or more amino acid modifications in the Fc region. The Fc region is said to comprise a (i) first mutation at E430, E345 or S440, with the proviso that the mutation in S440 is S440Y or S440W; and a (ii) second mutation at K322 or P329. The first
30 mutation is selected from the group consisting of: E430G, E345K, E430S, E430F, E430T, E345Q, E345R, E345Y, S440W and S440Y. The present invention does not include any of these substitutions.

WO2014/108198 discloses polypeptides such as antibodies comprising variant Fc regions having
35 one or more amino acid modifications resulting in increased complement-dependent cytotoxicity (CDC). The present invention does not include any of these substitutions.

WO2013/004842 describes polypeptides and related antibodies comprising a variant Fc domain for stabilising Fc:Fc interactions when the polypeptide(s), antibody or antibodies are bound to its target

on the cell surface for improved effector functions, such as CDC responses. The present invention does not include any of these substitutions.

Summary of the invention

5 The present invention provides polypeptides, antibodies, variants and antigen-binding fragments thereof having enhanced functional affinity compared to its parent polypeptide, antibody, variant and antigen-binding fragment thereof. The present invention also provides polypeptides, antibodies, variants and antigen-binding fragments thereof having enhanced cell killing compared to its parent polypeptide, antibody, variant and antigen-binding fragment thereof. Without being
10 limited to theory, it is believed that such modified polypeptides, antibodies, variants and antigen-binding fragments thereof are capable of more stable binding interactions between the Fc regions of two antibodies, thereby providing increased functional affinity of antibody-antigen-binding. Further, the inventors disclose a discontinuous region within the mIgG3 CH2 and CH3 domains that endows this isotype with increased functional affinity, on binding
15 repeating glycan antigen, as well as direct cytotoxicity on high-binding cancer cell lines. Transfer of the involved residues into the IgG1 isotype, creates an improved 'i'IgG1 with increased *in vitro* and *in vivo* anti-tumour activity.

By crosslinking chimeric mAbs with anti-human Ig anti-serum or by changing one key amino acid
20 residue we demonstrated that direct cell killing is directly related to functional affinity. Further, we recapitulated the functional affinity of our novel, mouse mAbs by introducing intermolecular cooperativity in the chimeric constructs. No one has mapped the mIgG3 cooperativity and the key amino acids that differ between IgG1 and mIgG3 have not been described previously. Thus, the inventors not only instigated the development of our series of anti-glycolipid mAbs but developed a
25 technology which has utility to improve the therapeutic index of any mAb.

Chimerisation of the mIgG3 mAbs onto a human IgG1 backbone coincided with a dramatic reduction in direct cytotoxicity, leading the inventors to hypothesize that this was the result of diminished intermolecular cooperativity. Consequently, the inventors looked to identify the key
30 residues within mIgG3 that are responsible for intermolecular cooperativity and transfer them into IgG1 in order to recapitulate the mIgG3-observed direct cytotoxic activity, thereby creating a chimeric IgG1 with superior clinical utility.

The creation of IgG1 anti-glycan mAbs with increased functional affinity and direct cytotoxic activity
35 through the transfer of selected mIgG3 constant region residues was undertaken by the inventors, utilising our panel or previously generated glycan targeting mAbs. mIgG3 contributing regions were identified through the creation of hybrid IgG1 constructs, containing mIgG3 CH1, CH2 or CH3 domains. Candidate residues were identified through screens based on increased direct cytotoxicity and functional affinity, when introduced into IgG1 (gain-of-function), and/or decreased
40 direct cytotoxicity and functional affinity when replaced by the respective IgG1 residues in mIgG3

(loss-of-function). Preliminary analyses ascertained that mlgG3 CH1 had a negligible contribution to the direct cytotoxicity ability as introducing mlgG3 CH1 into IgG1 did not lead to a significant increase in cytotoxicity. Conversely, introducing human IgG1 (hIgG1) CH1 into mlgG3, equally, did not instigate a significant reduction in killing activity. Next, in a gain-of-function approach, the mlgG3 CH2 and CH3 domains, separately, were introduced in IgG1. IgG1 containing murine CH3 exhibited a significant increase in cytotoxicity. Introducing murine CH2 into IgG1 led to small, but not significant, increase in killing activity. As a confirmation of the contributions made by both domains, the reverse strategy was followed whereby a loss of cytotoxicity activity was evaluated due to the introduction IgG1 CH2 or CH3 domains into mlgG3. This scenario led to a significant decrease in cytotoxicity for mlgG3 containing IgG1 CH3, corroborating the previous gain-of-function results. Importantly, this strategy also identified a small contribution by the murine CH2, as mlgG3 containing human CH2 exhibited a significant decrease in cytotoxicity activity.

According an aspect of the invention there is provided a modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residues of an Fc-region of an immunoglobulin and a binding region, wherein one or more residues of the Fc-region are modified to the corresponding residue from a mouse IgG3 antibody and wherein the modified IgG1 antibody or antigen-binding fragment thereof has enhanced functional affinity when compared to a corresponding IgG1 antibody or antigen binding fragment thereof comprising wildtype Fc-region residues.

In some aspects of the invention there is provided a modified IgG1 antibody or antigen-binding fragment thereof wherein the functional affinity of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 10% when compared to a corresponding IgG1 antibody or antigen binding fragment thereof comprising wildtype Fc-region residues.

In some aspects of the invention the functional affinity of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 100% when compared to a corresponding IgG1 antibody or antigen binding fragment thereof comprising wildtype Fc-region residues.

In some aspects of the invention the modified IgG1 antibody or antigen-binding fragment thereof has enhanced direct cell-killing when compared to a corresponding IgG1 antibody or antigen binding fragment thereof comprising wildtype Fc-region residues.

In some aspects of the invention the direct cell-killing of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 10% when compared to a corresponding IgG1 antibody or antigen binding fragment thereof comprising wildtype Fc-region residues.

- In some aspects of the invention the direct cell-killing of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 100% when compared to a corresponding IgG1 antibody or antigen binding fragment thereof comprising wildtype Fc-region residues.
- According to a first aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof wherein one or more residues of the CH2 and/or the CH3 domain are replaced with the corresponding residues from the mouse IgG3 CH2 and/or CH3 domain.
- According to a second aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof wherein the CH2 and/or the CH3 domain is replaced with a mouse IgG3 CH2 and/or CH3 domain.
- Further dissection of the combined CH2-CH3 region through subdomain analysis revealed full regain of mIgG3 direct cytotoxicity to IgG1 by transferring a discontinuous section comprising residues 286-306 and 339-378, containing elements of CH2 and CH3, 26 mIgG3 residues in total. The 26 residues are N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S. These residues are required for increased functional affinity through intermolecular cooperativity due to the combined effect of directly interacting as well as conformational residues, the latter potentially creating a permissive framework. A role for charge distribution patterns, notably in CH2, can also not be ruled out, as it has been shown to enhance mIgG3 binding to negatively charged multivalent antigen and is distinct from IgG1 (Klaus and Bereta 2018; Hovenden et al. 2013). Some of the residues involved in our approach lay within a larger consensus binding site for a number of natural binding partners such as protein A, protein G, rheumatoid factor and the neonatal FcRn (DeLano et al. 2000). However, none of our residues are directly involved in FcRn binding (Oganessian et al. 2014).
- According to the third aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof with modifications to one or more of the following residues of the Fc region N286, K288, K290, E294, Y300, V305, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.
- According to the fourth aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof with one or more of the following modifications to the Fc region N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.
- In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22,

23, 24, 25 or 26 residues selected from positions N286, K288, K290, E294, Y300, V305, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at all 26 residues.

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In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 modifications selected from N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A and A378S. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises all 26 modifications.

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A plethora of Fc engineering strategies, mostly to impact on mAb effector functions (ADCC and CDC), through modifying FcγR or C1q binding, as well as mAb half-life, via FcRn engagement, have been described in the literature (Wang, Mathieu, and Brezski 2018; Carter 2006). None of these describe the amino acids changes that form part of this patent. Additionally, crystal packing-induced mAb oligomerization through Fc:Fc interactions in a number of human mAb isotypes (Saphire et al. 2001) formed the basis of a recently described hexameric mAb platform for improved complement activation (WO2014/006217 A9; WO2018/083126 A1; WO2018/146317 A1, Ugurlar et al. 2018; de Jong et al. 2016; Diebolder et al. 2014). Efficient hexameric arrangement and C1q binding depended on the individual mutations (E345K or E430G) which are distinct from the amino acid changes which form the basis of this patent. Indeed, the ability to induce programmed cell death (i.e. direct cytotoxicity) by the type II CD20 mAb 11B8, through these mutations that induced a strong CDC ability, was not enhanced, suggesting this position was not responsible for direct cell killing.

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Most approved therapeutic mAbs are either humanised or fully human IgG antibodies with murine or chimeric antibodies carrying 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). The introduction of only 26 mIgG3 in IgG1 is unlikely to induce HAMA responses. However, they may create MHCII binding epitopes that have the potential to drive HAMA responses in certain patients. Immune Epitope Database (IEDB) analysis of the 26 residue-containing hybrid 88IgG1, revealed a cluster (cluster 1, residues 294-315) containing several potential high-scoring epitopes. Reverting of three mIgG3 back to IgG1 residues in cluster 1, at positions 294, 300 and 305, maintained the functional affinity and direct cytotoxicity. Importantly, modification of this 23 aa motif (N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378), which when modified to the corresponding mouse IgG3 residues are (N286T, K288W, K290Q, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S) induced direct cytotoxicity, cellular aggregation, pore formation and eventual cell lysis. The pore formation and eventual cell lysis share similar cellular

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disintegration features with necroptosis but cannot be distinguished from necrosis or secondary necrosis (Vanden Berghe et al. 2010). The eventual outcome from the released DAMPs - constitutive or induced as a result of activated stress pathways - depends on the cellular environment as well as the underlying signalling cascades, but collectively have the potential to create an inflammatory environment that may further enhance immune effector functions and/or instigate an adaptive immune response through cross-presentation of released tumour antigens (Yatim, Cullen, and Albert 2017; Galluzzi et al. 2017).

According to the fifth aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof with modifications to one or more of the following residues of the Fc region N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.

According to the sixth aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof with one or more of the following modifications to the Fc region N286T, K288W, K290Q, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.

In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 residues selected from positions N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at all 23 residues.

In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 modifications selected from N286T, K288W, K290Q, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises all 23 modifications.

This set of 23 modifications is exemplified for the 88 antibody in Figure 9A and 9B.

Further validation has been demonstrated by the introduction of the selected 23 mIgG3 residues into the sialyl-di-Lewis^a targeting 129 mAb, that has a more favourable normal tissue distribution whilst targeting a wide range of tumour tissue, notably over 70% of pancreatic, and over 30% of gastric and colorectal tumours, as well as over 20% of ovarian and non-small cell lung cancer tumours. Interestingly, the hybridoma-produced parental 129 mAb is a mIgG1 that lacks direct cytotoxic ability. Thus, the creation of an i129G1 mAb with significantly improved functional affinity,

through a slower dissociation rate, compared to 129IgG1, coinciding with nanomolar direct cell killing ability on COLO205 suggests that our approach may have broader applicability, as well as being relevant for immunomodulatory mAbs that rely on avidity effects (Wang, Mathieu, and Brezski 2018). The direct cell killing exerted by i129G1 manifested itself in a similar manner as for i88G1: mAb-induced cellular aggregation followed by pore formation and eventual cell lysis. The introduction of the 23 mlgG3 residues into i129G1 mAb had a mixed effect on effector functions: whilst ADCC was significantly reduced, but maintaining nanomolar EC₅₀, the CDC activity of i129G1 was significantly increased. This mirrored the results obtained with i88G1, albeit with a stronger reduction in ADCC for i129G1, suggesting that the nature of the glyco-target also affects ADCC potency: whereas the 88 mAb targets glycoproteins as well as glycolipids, the 129 mAb only targets glycoproteins. Remarkably, i129G1 displayed effective tumour control i129G1 exhibited significant tumour volume reduction in that was significantly better than 129IgG1, the latter exhibiting no significant tumour reduction. This indicates that the combined effector functions of 129IgG1 were unable to control tumour growth in this model, further emphasizing the value of having direct cytotoxic ability.

In order to evaluate the individual contribution of each of the 23 selected mG3 residues, a single revertant strategy based on i88G1 (example 4) was performed. Each of the 23 mG3 residues in i88G1, singly, were reverted to the hG1 residue and the resulting construct was analysed for direct cell killing this confirmed that the 23 aforementioned residues could be further reduced to 15 residues without significant loss of direct cell killing ability. Notably, six mlgG3 residues in i88G1 could be reverted to IgG1 residues without significant loss of direct cell killing. This was further exemplified using the 129 mAb where the introduction of 15 mlgG3 residues maintained nanomolar direct cell killing on COLO205.

Immunogenicity can be a major obstacle to successful therapy. Anti-drug antibodies may neutralise therapeutic function, influence pharmacokinetics and potentially lead to severe adverse effects. T cell epitopes, which contributes to immunogenicity risk, can be relatively accurately assessed using *in silico* tools. Immunoinformatic algorithms for identifying T-cell epitopes can be applied to assess and analyse whether an immunotherapy will cause unwanted immunogenicity. In order to assess the potential immunogenicity of our hybrid SD286 306+339 378 construct, created by the presence of 26 mlgG3 residues, we performed an *in silico* screen of the SD286 306+339 378 sequence for MHCII binding epitopes (Immune Epitope Database, IEDB). Class II restricted T helper cells are more relevant to the humoral immune response and predicted binding clusters have been shown to be strong indicators of T cell responses (Jawa et al. 2013). Two MHCII binding clusters, containing several high scoring binding epitopes, were identified: cluster 1 (residues 294 315) and cluster 2 (residues 365 393) (Figure 5/Figure 20). Reversion of three murine residues, 294 (A to E), 300 (F to Y) and 305 (A to V) back to human residues, within the cluster 1, produced a human sequence section to which individuals would have been tolerated. Similarly, reversal of two residues 351 (I to L) and 371 (N to G), within the 339 378 region, removed two MHCII binding cores.

Reversal of one in silico identified immunogenic cluster generates the lead candidate, improved 'i' 88G1, with robust cell killing, pore forming ability and sound immune effector functions.

To demonstrate the effect of introducing the selected residues was not restricted to anti-glycan antibodies, Trastuzumab (sold under the brand name Herceptin® among others) which targets the
5 growth factor receptor, HER2 was subjected to the transfer of the involved residues. The resultant iTrastuzumab had improved steady-state cell binding to moderate HER2-expressing cell lines, coinciding with increased PI uptake. Additionally it had improved affinity, through a slower dissociation rate, compared to wildtype.

According to the seventh aspect of the invention, there is provided a modified IgG1 antibody or
10 antigen-binding fragment thereof with modifications to one or more of the following residues of the Fc region N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378.

According to the eighth aspect of the invention, there is provided a modified IgG1 antibody or
15 antigen-binding fragment thereof with one or more of the following modifications to the Fc region N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S.

This set of 15 modifications is exemplified for antibodies 129 and 88 in Figures 9C and 9D.
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In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 residues selected from positions N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding
25 fragment thereof comprises modifications at all 15 residues.

In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 modifications selected from N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E,
30 D376A, A378S. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises all 15 modifications.

According to the ninth aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof with modifications to one or more of the following residues of the
35 Fc region Q342, P343, E345, N361, Q362, P374, D376.

According to the tenth aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof with one or more of the following modifications to the Fc region Q342R, P343A, E345T, N361K, Q362K, P374S, D376A.

This set of 7 modifications is exemplified for antibody 88 in Figure 9F.

5 In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at 1, 2, 3, 4, 5, 6 or 7 residues selected from positions Q342, P343, E345, N361, Q362, P374, D376. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises modifications at all 7 residues.

10 In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises 1, 2, 3, 4, 5, 6 or 7 modifications selected from Q342R, P343A, E345T, N361K, Q362K, P374S, D376A. In a preferred aspect of the invention the modified IgG1 antibody or antigen-binding fragment thereof comprises all 7 modifications.

15 In one aspect of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises one or more of the sequences disclosed in Figure 15. In one aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof comprising a heavy chain and a light chain sequence as disclosed in Figure 15.

20 In one aspect of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises one or more of the sequences disclosed in Figure 16. In one aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof comprising a heavy chain and a light chain sequence as disclosed in Figure 16.

25 In one aspect of the invention, the modified IgG1 antibody or antigen-binding fragment thereof comprises one or more of the sequences disclosed in Figure 17. In one aspect of the invention, there is provided a modified IgG1 antibody or antigen-binding fragment thereof comprising a heavy chain and a light chain sequence as disclosed in Figure 17.

30 In one aspect of the invention, the modified IgG1 antibody or antigen binding fragment thereof comprises one or more of the sequences disclosed in Figure 19. In one aspect of the invention, there is provided a modified IgG1 antibody or antigen binding fragment thereof comprising a heavy chain and a light chain sequence as disclosed in Figure 19.

35 In some aspects of the invention, the "corresponding IgG1 antibody or antigen-binding fragment thereof" refers to the unmodified (i.e. wildtype) antibody or antigen-binding fragment thereof which comprises the native residues without any changes to the corresponding mouse IgG3 residues. In some aspects of the invention, the corresponding IgG1 antibody or antigen-binding fragment thereof binds to the same target or targets (e.g. the same epitope) as the modified IgG1 antibody or antigen-binding fragment thereof. In some aspects of the invention the modified and
40 corresponding unmodified IgG1 antibody or antigen-binding fragment thereof comprise at least 1,

at least 2, at least 3, at least 4, at least 5 or at least 6 of the same CDR sequences (i.e. VHCDR1, VHCDR2, VHCDR3, VLCDR1, VLCDR2, VLCDR3). In some aspects of the invention the modified and corresponding unmodified IgG1 antibody or antigen-binding fragment thereof comprise the same binding region sequences. In some aspects of the invention the modified and corresponding unmodified IgG1 antibody or antigen-binding fragment thereof comprise the same variable region sequences (e.g. the same variable heavy chain sequence and/or the same variable light chain sequence).

In some aspects of the invention the "binding region" refers to the portion of the antibody or antigen-binding fragment thereof which is responsible for binding to the target antigen. For example it may comprise one constant and one variable domain of each of the heavy and the light chain. In some aspects of the invention, the binding region can refer to complementarity determining regions (CDR) sequences (i.e. VHCDR1, VHCDR2, VHCDR3, VLCDR1, VLCDR2, VLCDR3).

The structure of the IgG1 antibody or antigen-binding fragment thereof will generally be of an antibody heavy or light chain sequence or substantial portion thereof. The structures and locations of immunoglobulin domains may be determined by reference to <http://www.imgt.org/>.

Throughout the specification the residue numbering refers to the standardised IMGT system for the numbering of antibody sequences, as disclosed in Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, et al. IMGT, the international ImMunoGeneTics information system. *Nucleic acids research*. 2009;37:D1006-12. Other suitable numbering systems are known to the skilled person. Other suitable numbering systems may be used to identify corresponding residues between the modified IgG1 antibodies and antigen-binding fragments thereof or antigen-binding fragments thereof. Any numbering system which allows identification of corresponding residues is suitable for use with the present invention. The numbering system used herein is not limiting on the scope of the invention, but is used simply to identify the relevant residues which may be modified. The term "corresponding residue" is intended to mean the residue in the equivalent position, structurally or functionally, in the two or more antibodies or antigen-binding fragments thereof that are being compared. In some cases, corresponding residues may be identified by sequence alignment. In some cases, corresponding residues may be identified by structural comparison.

In some aspects of the invention the IgG1 antibody or antigen-binding fragment thereof comprising one or more residues of an Fc-region of an immunoglobulin comprises at least 10 amino acid residues of an Fc-region, at least 20 amino acid residues of an Fc-region, at least 30 amino acid residues of an Fc-region, at least 40 amino acid residues of an Fc-region, at least 50 amino acid residues of an Fc-region, at least 75 amino acid residues of an Fc-region, at least 100 amino acid residues of an Fc-region, at least 200 amino acid residues of an Fc-region, at least 300 amino acid

residues of an Fc-region, at least 400 amino acid residues of an Fc-region or at least 500 amino acid residues of an Fc-region. Preferably the IgG1 antibody or antigen-binding fragment thereof comprises the entire Fc-region of an immunoglobulin.

5 In some aspects of the invention, the one or more residues of the Fc region are selected from the CH2 and/or the CH3 domains. In some aspects the one or more residues are selected from the CH2 domain. In some aspects the one or more residues are selected from the CH3 domain.

10 In some aspects of the invention the functional affinity of a modified IgG1 antibody or antigen-binding fragment thereof and the corresponding wildtype IgG1 antibody or antigen-binding fragment thereof may be determined by Surface Plasmon Resonance (e.g. Biacore 3000/ T200, GE Healthcare), for example by injecting increasing concentrations (0.3nmol/L-200nmol/L) of an IgG1 antibody or antigen-binding fragment thereof across a CM5 chip comprising an appropriate ligand and fitting the data to an appropriate binding model using appropriate software (e.g. 15 BIAevaluation 4.1). Corresponding experiments using the same conditions can be conducted for the modified IgG1 antibody or antigen-binding fragment thereof. In some aspects of the invention, the modified IgG1 antibody or antigen-binding fragment thereof shows greater functional affinity to the corresponding wildtype IgG1 antibody or antigen-binding fragment thereof when the Surface Plasmon Resonance data indicates that the modified IgG1 antibody or antigen-binding fragment 20 thereof binds more tightly to the ligand-coated CM5 chip. Ligands comprise glycan-HSA and control conjugates for anti-glycan antibodies.

The Biacore CM5 chip, coated with an anti-his antibody, comprises carboxymethylated dextran covalently attached to a gold surface. Molecules are covalently coupled to the sensor surface via amine, thiol, aldehyde or carboxyl groups. Interactions involving small organic molecules, such as 25 drug candidates, through to large molecular assemblies or whole viruses can be studied. A high binding capacity gives a high response, advantageous for capture assays and for interactions involving small molecules. High surface stability provides accuracy and precision and allows repeated analysis on the same surface. Other suitable chips are known to the skilled person and the Surface Plasmon Resonance protocols can be adapted by standard techniques known in the 30 art.

In some aspects of the invention the direct cell killing of a modified IgG1 antibody or antigen-binding fragment thereof and the corresponding wildtype IgG1 antibody or antigen-binding fragment thereof may be determined by propidium iodide (PI) uptake, for example by incubating the IgG1 antibody or antigen-binding fragment thereof with COLO205 or HCT-15 cells (5×10^4) for 35 2 hours at 37°C followed by adding 1µg of PI for 30 minutes, resuspending the cells in PBS and processing the cells on a flow cytometer (e.g. Beckman Coulter FC-500 or MACSQuant 10) and analysing the results with appropriate software (e.g. WinMDI 2.9 or FlowJo v10). Corresponding experiments using the same conditions can be conducted for the modified IgG1 antibody or antigen-binding fragment thereof. In some aspects of the invention, the modified IgG1 antibody or

antigen-binding fragment thereof provides greater direct cell killing than the corresponding wildtype IgG1 antibody or antigen-binding fragment thereof when the PI uptake is greater when the cells are incubated with the modified IgG1 antibody or antigen-binding fragment thereof.

5 The improved functional properties of the modified IgG1 antibody or antigen-binding fragment thereof are measured relative to the corresponding functional properties of the wild type IgG1 antibody or antigen-binding fragment thereof that does not comprise the modified residues in the Fc-region. Since the improved functional properties are a relative measure, the precise method used to determine the functional affinity and/or direct cell killing, or any other functional property of the claimed IgG1 antibodies or antigen-binding fragments thereof, does not affect the relative
10 change in that functional property. Many suitable methods of measuring the functional properties of antibodies and antigen-binding fragments thereof are known to the skilled person. Any suitable method of measuring these functional properties may be used to measure the functional properties of the claimed modified IgG1 antibodies and antigen-binding fragments thereof or antigen-binding fragments thereof, provided the same method is applied to the corresponding wildtype IgG1
15 antibody or antigen-binding fragment thereof for the purposes of a fair comparison.

The creation of improved cancer glycan targeting mAbs, with enhanced functional affinity as well as direct cytotoxicity, through establishing intermolecular cooperativity binding, may lead to superior clinical utility. This approach may also have value for other mAbs targeting repeating or high-density antigen. Additionally, reinstating the unusual, proinflammatory cell killing mode
20 observed for many glycan-targeting mIgG3 mAbs, into the IgG1 framework, has the potential to be used in combination with other immunotherapies.

The modified IgG1 antibody of the present invention can be used in methods of diagnosis and treatment of tumours in human or animal subjects. When used in diagnosis, the modified IgG1 antibodies and antigen-binding fragments thereof of the invention may be labelled with a
25 detectable label, for example a radiolabel such as I or Tc, which may be attached to the modified IgG1 antibody 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. Although the modified IgG1 antibodies and antigen-binding
30 fragments thereof 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
35 modified IgG1 antibodies and antigen-binding fragments thereof may be attached or otherwise associated with chemotherapeutic or cytotoxic agents, such as maytansines (DMI and DM4), onides, auristatins, calicheamicin, duocamycin, doxorubicin 90 131 or radiolabels, such as Y or I. Furthermore, the modified IgG1 antibodies and antigen-binding fragments thereof 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 the modified IgG1 antibodies and antigen-binding fragments thereof 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
5 chemotherapeutic or cytotoxic agents including, 5-Fluorouracil, cisplatin, Mitomycin C, oxaliplatin and tamoxifen, which may operate synergistically with the modified IgG1 antibodies and antigen-binding fragments thereof 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.

Whilst not wishing to be bound by theory, the ability of the modified IgG1 antibodies and antigen-binding fragments thereof 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
15 consequence of the modified IgG1 antibodies and antigen-binding fragments thereof binding to cell surface receptor. The modified IgG1 antibodies and antigen-binding fragments thereof 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 modified IgG1 antibodies and antigen-binding fragments thereof.

The pharmaceutical composition may comprise, in addition to active ingredient, pharmaceutically acceptable excipient, diluent, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should 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
25 of administration, which may be oral, or by injection, *e.g.*, intravenous. Preferably the route of administration is 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
30 suitable routes of administration 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 suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable
35 solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or
40 other additives may be included, as required.

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 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 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 gamma ethyl-L-glutamate [43], poly(2-hydroxyethyl-methacrylate). Liposomes containing the polypeptides are prepared by well-known methods: (Eppstein et al. 1985; Hwang, Luk, and Beaumier 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 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.

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, 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 mentioned above can be found in Remington's Pharmaceutical Sciences 16 edition Oslo,A (ed) 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 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 $\ln M$ is normally sufficient. For example, a dose of 100 mg/m^2 of antibody provides a serum concentration of approximately 20 nM for approximately eight days. 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 glycan.

- 5 The dose of the composition will be dependent upon the properties of the modified IgG1 antibodies and antigen-binding fragments thereof, *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
- 10 of 3000g of antibody per patient per administration are preferred, although dosages may range from about 10µg to 6 mg 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.
- 15 The modified IgG1 antibodies and antigen-binding fragments thereof of the present invention may be generated wholly or partly by chemical synthesis. The modified IgG1 antibodies and antigen-binding fragments thereof 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) [46], in M.
- 20 Bodanzsky and A. 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.
- 25 Another convenient way of producing the modified IgG1 antibodies and antigen-binding fragments thereof according to the present invention is to express the nucleic acid encoding them, by use of nucleic acid in an expression system. The present invention further provides an isolated nucleic acid encoding the modified IgG1 antibodies and antigen-binding fragments
- 30 thereof of the present invention. Nucleic acid includes DNA and RNA. The skilled person will be able to determine substitutions, deletions and/or additions to such nucleic acids which will still provide the modified IgG1 antibodies and antigen-binding fragments thereof of the present invention.
- 35 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 the modified IgG1 antibodies and antigen-binding fragments thereof of the invention forms an aspect of the present invention, as does a method of

production of the modified IgG1 antibodies and antigen-binding fragments thereof which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression the modified IgG1 antibodies and antigen-binding fragments thereof may be isolated and/or purified using any suitable technique, then used as appropriate. 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 (Pluckthun 1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of the modified IgG1 antibodies and antigen-binding fragments thereof, see for recent review, for example (Reff 1993; Trill, Shatzman, and Ganguly 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, Fritsch, and Maniatis 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 et al. 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. In one embodiment, the nucleic acid of the invention is 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 the modified IgG1 antibodies and antigen-binding fragments thereof as above.

5 The fragment crystallizable region (Fc region) is the tail region of an antibody that interacts with cell surface receptors called Fc receptors and some proteins of the complement system. This property allows antibodies to activate the immune system. In IgG antibody isotypes, the Fc region is composed of two identical protein fragments, derived from the second and third constant domains of the antibody's two heavy chains. The Fc regions of IgGs comprise a highly conserved N-glycosylation site.

10 The Fc (Fragment, crystallizable) of an IgG consists of a paired set of antibody HC domains, each of which has a CH2 fused to a CH3, which form a structure of about 50 kDa. The name "Fragment, crystallizable" (Fc) comes from the fact that after cleavage of serum-derived myeloma IgG fractions with papain, the only fragment that could be crystallized was the paired CH2-CH3 fragment.

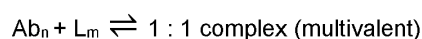
15 Within the Fc, the two CH3 domains bind each other tightly, whereas the two CH2 domains have no direct protein-protein contact with one another. An oligosaccharide is bound to asparagine-297 (N297) within each of the two CH2 domains, filling part of the space between the two CH2s. In some crystal structures, hydrogen bonding has been observed between the two carbohydrate chains, directly and through bridging water molecules. While the antibody
20 appears to be a highly segmented molecule, it has been demonstrated that the structure of the Fc can impact the binding of the FAbs to the targeted antigens and, similarly, that the content of the variable chain in the FAbs can impact binding of the Fc to various receptors. Recently circular dichroism studies have confirmed significant structural coupling between the FAb arms and the Fc of the IgG. Thus the IgG molecule is a highly complex molecule in which the different
25 domains significantly interact, even at long distances.

While bi-/multivalent antibodies comprise binding domains that interact with epitopes, synthetic bi-/multivalent ligands comprise pharmacophores that interact with target sites. The term 'functional affinity' can also be used to designate a bi-/multivalency-related increase in apparent
30 affinity. This term is synonymous with 'avidity'.

Avidity refers to the accumulated strength of multiple affinities of individual non-covalent binding interactions, such as between a protein receptor and its ligand, and can also be referred to as 'functional affinity'. As such, avidity is distinct from intrinsic affinity, which describes the strength of
35 a single interaction. However, because individual binding events increase the likelihood of other interactions to occur (i.e. increase the local concentration of each binding partner in proximity to the binding site), avidity should not be thought of as the mere sum of constituent affinities but as the combined effect of all affinities participating in the biomolecular interaction. The utility of the distinction between "intrinsic affinity" and "functional affinity" arises from the different emphasis

involved in each term. The former is most useful when the structural relationship between the antibody combining site and the complementary region of the ligand is under scrutiny or when kinetic mechanisms of the specific interaction are under investigation. On the other hand, the latter is particularly significant when the quantitative measurement of the enhancement of affinity is being examined, as in the present invention.

Both 'functional affinity' and 'intrinsic affinity' refer to formally identical reversible processes as follows:



where F_1 is a monovalent antibody fragment, L_1 a monovalent ligand, Ab_n is a multivalent antibody, and L_m is a multivalent ligand with m groups. In each instance, two kinetic units combine reversibly to form one unit and each process can, in principle, be characterized by an association constant. Since the association constant is a measure of the thermodynamic affinity both processes can therefore be assigned quantitative values of the affinity.

Fragments of antibodies and antigen-binding molecules

The antigen-binding molecule of the invention can be a fragment of an antibody, specifically an antigen-binding fragment of an antibody. The antigen-binding fragments comprise one or more antigen-binding regions. 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, E.S. et al., Nature 341:544-546 (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 et al., Science 242:423-426 (1988); Huston et al., PNAS USA 85:5879-5883 (1988)); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993)). Typically, the fragment is a Fab, F(ab')₂ or Fv fragment or an scFv molecule.

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).

5

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Hollinger & Winter, *Current Opinion Biotechnol.* 4:446-449 (1993)), e.g. prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned below. 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 et al., *EMBO Journal* 10:3655-3659 (1991).

10

15

A bispecific antibody is one which can bind to two target molecules simultaneously, such as two antigens or two epitopes. Bispecific antibodies may also be referred to as dual binding antibodies. Examples of bispecific antibody formats include, but are not limited to; (mAb)₂, Fcab, F(mAb')₂, quadromas, scFv (single chain variable fragments), bsDb (bispecific diabodies), scBsDb (single chain bispecific diabodies), BiTE (bispecific T cell engagers), DART (dual affinity re-targeting antibodies), charge pairs, tandem antibodies, tandem scFv-Fc, Fab-scFv-Fc, Fab-scFv, minibodies, zybodies, DNL-F(ab)₃ (dock-and-lock trivalent Fabs), bssdAb (bispecific single domain antibodies) and knobs-in-holes.

20

25

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.

30

In some aspects of the invention, it may be desirable to generate multispecific (e.g. bispecific) anti-target antibody having binding specificities for at least two different epitopes of the same or different molecules. Exemplary bispecific antibodies may bind to two different epitopes of the target molecule. Alternatively, a target-specific antibody arm may be combined with an arm which binds to a cell surface molecule, such as a T-cell receptor molecule (e.g., CD3). Exemplary bispecific antibodies of the invention can bind an antigen from a T-cell receptor molecule (e.g. CD3) and any other target.

35

The modified IgG1 antibodies and antigen-binding fragments thereof of the present invention

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). Modified IgG1 antibodies and antigen-binding fragments thereof of the present invention of the invention may be humanised, chimeric or veneered antibodies, or may be non-human antibodies of any species. In a preferred embodiment the modified IgG1 antibodies and antigen-binding fragments thereof of the present invention are human or humanized.

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.

Variants

The present invention also extends to variants of any peptide sequences disclosed herein. As used herein the term "variant" relates to proteins that have a similar amino acid sequence and/or that retain the same function. For instance, the term "variant" encompasses proteins or polypeptides which include one or more amino acid additions, deletions, substitutions or the like. An example of a variant of the present invention is a protein comprising a peptide as defined below, apart from the substitution of one or more amino acids with one or more other amino acids. Amino acid substitutions may be made to, for example, reduce or eliminate liabilities in the amino acid sequences. Alternatively, amino acid substitutions may be made to improve antigen affinity or to humanise or deimmunise the antibodies, if required. Affinity matured variants, humanised variants and deimmunised variants of the specified antibodies are provided herein, as well as variants comprising amino acid substitutions to reduce or eliminate any liabilities in the sequences of the antibodies.

As noted above, in some embodiments, any substitutions may occur only in the framework regions. In such embodiments, the original CDR sequences are retained, but variation may occur in one or more framework regions.

Variant antigen-binding molecules having the one or more amino acid substitutions may retain the functional activity (for example EC₅₀, IC₅₀, IC₉₀, K_d, functional affinity and/or direct cell killing) of the antigen-binding molecule from which the variant antigen-binding molecule is derived. Variant antigen-binding molecules of the invention can be used and formulated in the same ways as described for the antigen-binding molecules from which they are derived.

Substitutions

The skilled person is aware that various amino acids have similar properties. One or more such amino acids of a substance can often be substituted by one or more other such amino acids

without eliminating a desired activity of that substance.

Thus, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi- conservative" amino acid substitutions.

Using the three letter and one letter codes the naturally occurring amino acids may be referred to as follows: glycine (G or Gly), alanine (A or Ala), valine (V or Val), leucine (L or Leu), isoleucine (I or Ile), proline (P or Pro), phenylalanine (F or Phe), tyrosine (Y or Tyr), tryptophan (W or Trp), lysine (K or Lys), arginine (R or Arg), histidine (H or His), aspartic acid (D or Asp), glutamic acid (E or Glu), asparagine (N or Asn), glutamine (Q or Gln), cysteine (C or Cys), methionine (M or Met), serine (S or Ser) and Threonine (T or Thr). Where a residue may be aspartic acid or asparagine, the symbols Asx or B may be used. Where a residue may be glutamic acid or glutamine, the symbols Glx or Z may be used. References to aspartic acid include aspartate, and glutamic acid include glutamate, unless the context specifies otherwise.

Amino acid deletions or insertions can also be made relative to the amino acid sequence for the fusion protein referred to below. Thus, for example, amino acids which do not have a substantial effect on the activity of the polypeptide, or at least which do not eliminate such activity, can be deleted. Such deletions can be advantageous since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining activity. This can enable the amount of polypeptide required for a particular purpose to be reduced - for example, dosage levels can be reduced.

In some embodiments, the following amino acids can be exchange for each other for conservative amino acid substitutions:

Class	Exchangeable amino acids
Aliphatic	Glycine, Alanine, Valine,

	Leucine, Isoleucine
Hydroxyl or Sulfur/Selenium-containing	Serine, Cysteine, Threonine, Methionine
Aromatic	Phenylalanine, Tyrosine, Tryptophan
Basic	Histidine, Lysine, Arginine
Acidic and their Amide	Aspartate, Glutamate, Asparagine, Glutamine

Therefore, references to “conservative” amino acid substitutions refer to amino acid substitutions in which one or more of the amino acids in the sequence of the antibody (e.g. in the CDRs or in the VH or VL sequences) is substituted with another amino acid in the same class as indicated above. Conservative amino acid substitutions may be preferred in the CDR regions to minimise adverse effects on the function of the antibody. However, conservative amino acid substitutions may also occur in the framework regions.

Amino acid changes relative to the sequence given below can be made using any suitable technique e.g. by using site-directed mutagenesis or solid-state synthesis.

It should be appreciated that amino acid substitutions or insertions within the scope of the present invention can be made using naturally occurring or non-naturally occurring amino acids, although naturally occurring amino acids may be preferred. Whether or not natural or synthetic amino acids are used, it may be preferred that only L- amino acids are present.

Manufacturing liabilities

Therapeutic proteins such as antibodies are heterogenous and complex by nature due to chemical modifications and post-translational modifications (PTMs). Modifications can be caused by a number of factors such as the host cell system, processes used in manufacture or conditions during storage or manufacture. Modifications can relate to the chemical stability of the molecule itself or the aggregation potential and the effect this has on intrinsic physical stability of the antibody. Amino acid motifs or residues in a given antibody sequence that may undergo spontaneous modification during manufacture or storage are referred to as liabilities. Accordingly, mutations may be made to the antibody sequence to address the liabilities to reduce the susceptibility of the antibody to modification and degradation.

Such modifications as a result of liabilities in the antibody sequences may include glycosylation, deamidation, oxidation and variations of C- and N- termini. Such modifications may arise during manufacture. Certain residues and structural or sequence motifs are more liable to certain

modifications. Examples of such liabilities to modification include Asn N-linked glycosylation, Ser/Thr O-linked glycosylation, Asn deamidation, Asp isomerisation/fragmentation, Lys glycation, Met/Trp oxidation, free thiol groups, pyro-glutamates, C-terminal Lys.

5 A skilled person is aware that computational tools can be used to predict and identify structural and sequence liabilities which could potentially result in modifications. To minimise the occurrence of modifications alterations to the manufacturing process can be made. Protein engineering may also be considered to reduce the risk. For example, selective mutation of these liabilities can help to identify and reduce the risk of a modification endangering the stability of an antibody.

10 Aspartic acid residues (Asp) may undergo spontaneous modification. Asp containing motifs, such as Asp-Gly sequences may undergo spontaneous isomerization to form isoaspartic acid. Formation of isoaspartate may debilitate or completely abrogate the binding of the antibody.

15 This is of additional importance if the Asp residue appears in the CDR of an antibody.

Aspartic acid residues (Asp) can therefore be substituted with any naturally occurring amino acid to reduce this liability to modification. Optionally, aspartic acid residues (Asp) can be substituted with alanine (Ala), glutamine (Gln) or glutamic acid (Glu) to reduce this liability to modification. Optimization of production/formulation can also be investigated to reduce isomerization. Alternatively, Asp-Gly motifs may be modified by substituting the glycine residue with another naturally occurring amino acid to inhibit deamidation, rather than by substitution of the Asp residue.

20 Aspartic acid residues (Asp) can therefore be substituted with any naturally occurring amino acid to reduce this liability to modification. Optionally, aspartic acid residues (Asp) can be substituted with alanine (Ala), glutamine (Gln) or glutamic acid (Glu) to reduce this liability to modification. Optimization of production/formulation can also be investigated to reduce isomerization. Alternatively, Asp-Gly motifs may be modified by substituting the glycine residue with another naturally occurring amino acid to inhibit deamidation, rather than by substitution of the Asp residue.

25 Methionine residues (Met) may undergo spontaneous modification. The presence of methionine (Met) in a CDR, especially if exposed to solvent, can create a problem if the methionine is oxidized and this interferes with binding. Methionine residues can therefore be substituted with any other naturally occurring amino acid to reduce this liability to modification. Methionine residues may preferably be substituted with Ala or Leu. Optimization of production/formulation can also be investigated to reduce oxidation.

30 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.

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Brief description of the drawings

The following detailed description, given by way of example, but not intended to limit the invention solely to the specific embodiments described, may best be understood in conjunction with the accompanying drawings.

Figure 1. Maintenance of cancer cell binding, but significantly decreased direct cytotoxicity of 88IgG1 compared to 88mIgG3 and parental hybridoma mAb. A. Comparable HCT15 and COLO205 cell binding analysis by 88IgG1, 88mIgG3 and FG88 (hybridoma mAb); B. significantly reduced direct cytotoxicity (PI uptake) on HCT15 by 88IgG1 compared to parental 88mIgG3 and FG88; significantly reduced proliferation inhibition by 88IgG1 compared to parental 88mIgG3 and FG88 on COLO205 (C) and HCT15 (D). Significance was deduced from two-way ANOVA with Dunnett's corrections for multiple comparisons.

Figure 2. mIgG3 CH3 and to a lesser extent CH2 contribute to the direct cytotoxicity and improved functional affinity. Constant domain shuffling suggests no significant contribution by CH1 to direct cytotoxicity (PI uptake, HCT15, Panel A; proliferation inhibition on COLO205, panel B). In contrast, CH3 (1m3 and 3h3) contributes significantly to direct cytotoxicity, with a minor contribution by mCH2 only evident in a loss-of-function approach (3h2) (PI uptake, HCT15, Panel C; proliferation inhibition, COLO205, panel D). Significantly increased functional affinity and decreased off-rate by 1m3; with significantly decreased functional affinity and increased off-rate by 3h2 and more pronounced by 3h3, confirming the major CH3 and minor CH2 contributions (Panel E,F). Significance versus respective parental constructs was deduced from two-way ANOVA (direct cytotoxicity) or one-way ANOVA (functional affinity, off-rate), with Dunnett's corrections for multiple comparisons.

Figure 3. SD286-397 encompassing the CH2:CH3 junction underlies mIgG3 direct cytotoxicity and improved functional affinity. Subdomain divisions of CH2CH3 identify two regions that do not contribute: SD232-294 and SD390-447, as well as two regions that significantly contribute to direct cytotoxicity and functional affinity: SD286-345 (CH2) as well as SD339-397 (CH3). Significantly increased PI uptake by both constructs and the combination (SD286-397) compared to 88IgG1 on HCT15 (Panel A) and COLO205 (Panel B). Significantly increased proliferation inhibition by both constructs and the combination compared to 88IgG1 on COLO205 (Panel C) and HCT15 (Panel D). Significantly increased functional affinity (SPR), resulting mainly from reduced off-rates by the two aforementioned constructs (Panels E and F, respectively). Significantly reduced CDC activity (HCT15) by SD339-397 compared to 88IgG1 (Panel G); maintenance of ADCC activity (COLO205) by the aforementioned constructs (Panel H). Significance versus respective parental constructs was deduced from two-way ANOVA (direct cytotoxicity) or one-way ANOVA (functional affinity, off-rate, effector functions), with Dunnett's corrections for multiple comparisons.

Figure 4. Discontinuous region consisting of 286-306 combined with 339-378 imparts direct cytotoxicity and enhanced functional affinity, whilst maintaining immune effector functions. Significantly increased PI uptake (Panel A) and proliferation inhibition (Panel B) by SD339-378 compared to 88IgG1 on HCT15. Significantly reduced PI uptake by SD307-345 compared to

SD286-345, suggesting a contribution by SD286-306 (Panel C). Significantly increased proliferation inhibition by the combination of SD286-306+339-378 compared to 88IgG1 on HCT15 (Panel D) and COLO205 (Panel E), as well as PI uptake on COLO205 (Panel F). Significantly increased functional affinity (SPR) by SD286-306+339-378 compared to 88IgG1 (Panel G). Maintenance of CDC activity on HCT15 (Panel H) and ADCC on COLO205 (Panel I) by SD286-306+339-378 compared to 88IgG1. Significance versus respective parental constructs was deduced from two-way ANOVA (direct cytotoxicity) or one-way ANOVA (functional affinity, and effector functions), with Dunnett's corrections for multiple comparisons.

Figure 5. MHCII binding clusters containing several high-scoring binding epitopes. Cluster 1 consists of residues 294-315 and cluster 2, residues 365-393.

Figure 6. i88G1 with direct cytotoxicity and enhanced functional affinity, whilst maintaining immune effector functions, exhibits pore forming ability. Significantly increased proliferation inhibition on HCT15 (Panel A) and COLO205 (Panel B) by i88G1 compared to 88IgG1. Significantly increased functional affinity (SPR) by i88G1 compared to 88IgG1 (Panel C). Slight but significant reduction in ADCC by i88G1 compared to 88IgG1 on COLO205 (Panel D) and significantly increased CDC activity by i88G1 compared to 88IgG1 on HCT15 (Panel E). Evidence of cellular detachment, aggregation and pore forming ability by i88G1 on HCT15 (Panel F).

Figure 7. i129G1 with direct cytotoxicity and enhanced functional affinity, whilst maintaining immune effector functions, exhibits pore forming ability and *in vivo* tumour control. Significantly increased proliferation inhibition (Panel A) and PI uptake (Panel B) on COLO205 (Panel A) by i129G1 compared to 129IgG1. Significantly increased functional affinity (SPR) by i129G1 compared to 129IgG1 (Panel C). i129G1 maintains some ADCC activity on COLO205, but significantly reduced compared to h129IgG1 (Panel D). CDC activity by i129G1 on COLO205 is significantly increased compared to 129IgG1 (Panel E). Evidence of cellular detachment, aggregation and pore forming ability by i129G1 on COLO205 (Panel F). Significant *in vivo* tumour control by i129G1 compared to vehicle control and compared to 129IgG1 in a COLO205 xenograft model (Balb/c mice) (Panel G). No significant effect on mean body weight during the course of the mouse study (Panel H). Significance versus respective parental constructs was deduced from two-way ANOVA (direct cytotoxicity, and *in vivo* tumour control) or one-way ANOVA (functional affinity and effector functions), with Dunnett's corrections for multiple comparisons.

Figure 8. i27G1 exhibits significantly improved direct cytotoxicity and functional affinity compared to 27IgG1, whilst maintaining immune effector functions Significantly increased proliferation inhibition on MCF7 (Panel A) and AGS (Panel B) by i27G1 compared to 27IgG1. Significantly increased functional affinity (SPR) by i27G1 compared to 27IgG1 (Panel C). i27G1 maintains equivalent ADCC (Panel D) as well as CDC activity (panel E) on MCF7. Significance

versus parental constructs was deduced from two-way ANOVA (direct cytotoxicity) or one-way ANOVA (functional affinity) with Dunnett's corrections for multiple comparisons.

Figure 9. Single revertant analysis reveals a minimum of 15 residues are required to maintain direct cell killing. Direct cytotoxicity of 23 single i88G1 revertants on COLO205 (Panel A) and HCT15 (Panel B). Normalised cell killing (relative to i88G1) demonstrates the need for an increased number of mG3 residues to maintain direct cytotoxicity on HCT15 (15 residues), compared to COLO205 (seven residues). Improved constructs with 15 mG3 residues (i88G1v2, panels C and E and i129G1v2, Panel D) maintain equivalent direct cytotoxicity on COLO205 and HCT15 compared to parental constructs with 23 mG3 residues, i88G1 and i129G1, respectively.

Figure 10. 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 Lewis^a mAb, 505/4, or W6/32 which recognises HLA.

Figure 11. 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.

Figure 12A. 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 12B:** 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 12C:** PI uptake mediated by FG27.10, FG27.18, CH27 IgG2, CH27 IgG1 on C170 cells.

Figure 13. Binding of FG27.10, FG27.18, ch27IgG1 and the humanised variant hLewAC to a panel of cell lines: Figure 13A, HCT15; Figure 13B, AGS; Figure 13C, OVCAR3; Figure 13D, H322, and Figure 13E, MCF7, and SPR analysis for FG27.10 (Figure 13F) and FG27.18 (Figure 13G).

Figure 14. i27G1 exhibits significantly improved direct cytotoxicity and functional affinity compared to 27IgG1, whilst maintaining immune effector functions. Significantly increased proliferation inhibition on MCF7 (Panel i) and AGS (Panel ii) by i27G1 compared to 27IgG1. Significantly increased functional affinity (SPR) by i27G1 compared to 27IgG1 (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-way ANOVA (direct cytotoxicity) or one-way ANOVA (functional affinity) with Dunnett's corrections for multiple comparisons.

Figure 15. DNA and protein antibody 88 antibody sequences including "improved" versions 1-3.

Figure 16. DNA and protein antibody 129 antibody sequences including "improved" versions 1-3.

Figure 17. DNA and protein antibody 27 antibody sequences including "improved" version 1.

Figure 18. iTrastuzumab (iTv1 and iTv2) exhibit improved functional affinity compared to wild-type Trastuzumab (WT) in a ligand density-dependent manner (Biacore T200 analysis, Panel A). Cell binding overview of Trastuzumab constructs on a range of high-binding (SKBR3, BT474) and low-to-moderate binding cell lines (MDA-MB231 and SKOV3) (Panel B). Significantly improved direct cytotoxicity (iTv2) on MDA-MB231 compared to wild-type Trastuzumab (Panel C). Enhanced PI uptake on BT474 cells (reflecting membrane damage) by iTv1 and iTv2 (highest concentration) compared to wild-type Trastuzumab (Panel D). Improved solid-phase Her2 binding by directly labelled iTv2 compared to wild-type trastuzumab (Panel E). Significance versus parental constructs was deduced from two-way ANOVA (direct cytotoxicity) with Dunnett's corrections for multiple comparisons.

Figure 19. Protein antibody Trastuzumab antibody sequences including "improved" versions 1 & 2.

Figure 20. IEDB screen for MHC class II binding epitopes. Top, sequence alignment (ClustalW) of hlgG1 and mlgG3 sequences encompassing SD286-306+339-378. The combined subdomains, 286-306 and 339-378 are boxed; clusters 1 and 2, identified by IEDB analysis are represented by arrows. Below, IEDB analysis table with MHCII binding epitopes, DR alleles and scores in the middle column. The right hand column lists residues changes (mlgG3 -> hlgG1, underlined) that would resolve immunogenicity. DI, de-immunized construct.

Detailed description of the invention

In describing the embodiments of the invention, the terminology is not intended to be limited to the specific terms so selected, and it is understood that each specific term includes all technical equivalents which operate in a similar manner to accomplish a similar purpose.

The term 'immunoglobulin' or Ig, refers to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains and one pair of heavy (H) chains, all four potentially inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterised. See for instance Fundamental Immunology Ch.7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)).

Methods

Materials, cells and antibodies

All cancer cell lines: COLO205, HCT15 (C170, C170HM2), AGS, COLO201, ST16, MCF7, OVCAR3, H322, OVCA4, LoVo, MKN45, 791T, BT474, MDA-MB231, SKBR3, SKOV3, and 791T, as well as the murine myeloma NS0 cell line were purchased from ATCC (Virginia, USA). All cell lines were authenticated using short tandem repeat profiling. Human serum albumin (HSA)-APD- sialyl-Lewis^a and HSA-APD-Lewis^a were from IsoSepAB (Sweden). Erb2-His was sourced from Abcam as well as Stratech. Cell lines were maintained in RPMI medium 1640 (Sigma), or DMEM high glucose supplemented with 10% fetal calf serum, L-glutamine (2mM) and sodium bicarbonate-buffered. Parental murine FG88 and FG129 mAbs were generated as previously described (Chua et al. 2015).

Cloning of modified mAb constructs

In order to create chimeric IgG1 variants of our hybridoma-produced mAbs (FG88.2 and FG129), the heavy chain and light chain variable regions encoding the respective mAbs were introduced into the pDCOrig vector using the restriction enzymes BamHI/BsiWI (light chain locus) or HindIII/AfeI (heavy chain locus) (Metheringham et al. 2009). The synthetic heavy chain constant regions, including full mIgG3 constant regions as well as interchanged mIgG3-IgG1 domains and single residue changes, were designed and ordered from Eurofins MWG (Ebersberg, Germany). Typically, this involved a 1054bp cassette, stretching from the AfeI restriction site at the JH/CH junction to an XbaI site 3' to the CH stop codon. Synthetic genes were supplied in proprietary Eurofins vectors. After maxiprep (plasmid maxi kit, Qiagen), 15µg of plasmid DNA was digested with AfeI and XbaI (both NEB) and the insert gel purified (QIAquick gel extraction kit, Qiagen). This insert was introduced into AfeI/XbaI digested vector pOrigHiB (Metheringham et al. 2009) by ligation (T4 DNA ligase, NEB, following manufacturer's recommendations). Following sequence confirmation and maxiprep, 15µg of plasmid DNA was digested with AfeI and AvrII (both NEB) and the insert gel purified. This insert was then introduced into AfeI/AvrII digested vector pDCOrig by ligation.

HEK293 transfection and mAb purification

mAb constructs were obtained following transient transfections of Expi293F™ cells using the ExpiFectamine™ 293 Transfection kit (Gibco, LifeTechnologies). Briefly, HEK293 cells in suspension (100ml, 2x10⁶/ml) were transfected with 100µg DNA and conditioned medium harvested at day seven post-transfection. Conditioned transfection supernatant was filtered through 0.22µm bottle top filters (Merck Millipore) and sodium azide added to a final concentration of 0.2% (w/v). Antibody was purified on protein G columns (HiTrap ProteinG HP, GE Healthcare) using an AKTA FPLC (GE Healthcare). Columns were washed with PBS/Tris buffer (PBS with 50mM Tris/HCl, pH7.0) before antibody elution with a rapid (2ml) gradient into 100mM glycine, pH12 (supplemented with 0.05% v/v Tween 20), collecting 2ml fractions. Fractions containing mAb

were pooled and neutralized (using 1M HCl) and the concentration determined. All transiently expressed mAb constructs were then analysed for cell binding, compared to the parental mAbs, using flow cytometry as a read-out for correct folding of the mAb constructs (data not shown).

5 **Indirect immunofluorescence and flow cytometry**

Cancer cells (1×10^5) were incubated with primary mAbs (at 33.3nmol/L or titrated) for 1h at 4°C, as previously described (Chua et al. 2015) followed by 1h incubation at 4°C with anti-mouse/anti-human FITC labelled secondary antibody, and fixing in 0.4% formaldehyde. Stained samples were analysed on a MACSQuant 10 flow cytometer and analysed using FlowJo v10.

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Functional affinity determination

The kinetic parameters of the 88 and 129 mAbs binding to Lewis^a - or sialyl-Lewis^a -APD-HSA were determined by Surface Plasmon Resonance (SPR, Biacore 3000, GE Healthcare). Increasing concentrations (0.3nmol/L-200nmol/L) of mAb were injected across a CM5 chip and data were fitted to a heterogeneous ligand binding model using BIAevaluation 4.1. The chip contained four cells, two of which, HSA-coated (in-line reference cells), the other two were coated with low (30-80 response units (RU)) and high amounts (360-390 RU) of the respective glycan-APD-HSA.

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The kinetic parameters of Trastuzumab antibody versus Trastuzumab engineered antibody variants binding to its ligand, HER2 was determined by SPR (Biacore T200, Cytiva, formerly GE Healthcare). Increasing concentrations (90.0 nM to 0.37 nM of antibody were injected across a CM5 chip coated with anti-His antibody and captured His-tagged HER2

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***In vitro* cytotoxicity**

PI uptake as well as proliferation inhibition was performed to analyse the direct cytotoxic effect of the mAbs. COLO205, HCT-15 or BT474 cells (5×10^4) were incubated with mAbs for 2 hours at 37°C followed by the addition of 1µg of PI for 30 minutes. Cells were resuspended in PBS and run on a Beckman Coulter FC-500 or on a MACSQuant 10 flow cytometer and analysed with WinMDI 2.9 or FlowJo v10 software, respectively.

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Proliferation inhibition by the constructs was assessed by using the water-soluble tetrazolium salt WST-8 (CCK8 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 (1000-2000 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).

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Scanning electron microscopy

HCT-15 or COLO205 cells (1×10^5) were grown on sterile coverslips for 24 hours prior to mAb (0.2 $\mu\text{mol/L}$) addition for 18 hours at 37°C. Controls included medium alone and 0.5% (v/v) hydrogen peroxide (H_2O_2) (Sigma). Cells were washed with pre-warmed 0.1 M sodium cacodylate buffer pH7.4 (SDB) and fixed with 12.5% (v/v) glutaraldehyde for 24 hours. Fixed cells were washed twice with SDB and post-fixed with 1% (v/v) osmium tetroxide (pH 7.4) for 45 minutes. After a final wash with H_2O , the cells were dehydrated in increasing concentrations of ethanol and exposed to critical point drying, before sputtering with gold, prior to SEM analysis (JSM-840 SEM, JEOL).

In vivo model

The study was conducted by CrownBio UK under a UK Home Office Licence in accordance with NCRI, LASA and FELAS guidelines. Subcutaneous tumours of a human colorectal adenocarcinoma model of COLO 205 were established in age-matched female BALB/c nude (Charles River, UK) mice via injection of 5×10^6 viable cells in 0.1ml serum free RPMI:Matrigel (1:1) into the left flank of each mouse. Mice ($n=10$) were randomly allocated to treatment groups based on their mean tumour volume ($\sim 103\text{mm}^3 \pm 13\text{mm}^3$) on study day 6 and dosed intravenously (i.v.), biweekly, with mAbs (0.1 mg) or vehicle (PBS, 100 μl) up until week 5. Body weight and tumour volume were assessed three times weekly and reduction in tumour volume analysed statistically using a two-way ANOVA test with Bonferroni's post-test (interaction factors; GraphPad Prism v 7.4 (GraphPad Inc, La Jolla, CA)).

Examples

The present invention will now be described further with reference to the following examples and the accompanying drawings.

Example 1. m88G3 exhibits avid glycan binding as well as direct cytotoxicity in the absence of complement and immune effector cells, both of which are reduced upon chimerisation to 88IgG1.

It has previously shown that the hybridoma-produced mIgG3 mAb FG88.2 exerts a direct cytotoxic effect on high-binding cancer cell lines, such as COLO205 and HCT15, in the absence of complement or effector cells (Chua et al. 2015). This direct cytotoxicity involved mAb-induced cellular aggregation, proliferation inhibition as well as irregular pore formation through an oncolytic mechanism. We subsequently created a chimeric, HEK293-expressed, IgG1 mAb, 88IgG1, for clinical exploitation. 88IgG1 maintained equivalent HCT15 and COLO205 cancer cell binding levels (Figure 1A), compared to the hybridoma-produced FG88.2, as well as the HEK293-expressed 88mIgG3. The latter mAb was generated to rule out expression system related-effects such as differential Fc glycosylation, due to the use of murine hybridoma cells versus HEK293 cells. Surprisingly, 88IgG1, exhibited significantly reduced direct cytotoxicity on COLO205 and HCT15, across two functional assays, PI uptake and proliferation inhibition, compared to 88mIgG3 (Figure 1B-D). 88mIgG3 also displayed a modest reduction in direct

cytotoxicity compared to the hybridoma-produced FG88.2, suggesting that differential glycosylation of the Fc region by the two expression settings (mouse hybridoma versus HEK293 cells) may contribute to the effect. This suggests the direct cell killing was related to the kinetic binding of the different isotypes. Consequently, the kinetic binding of our isotype-switched mAbs was analysed on a Lewis^a-APD-HSA coated chip using SPR (Table 1). FG88.2 displayed avid Lewis^a-APD-HSA binding with fast apparent on-rates ($k_{on} \sim 10^4$ 1/smol/L) and very slow off rates ($k_{off} \sim 10^{-6}$ 1/s) on the high-density flow cell. The HEK293-produced 88mIgG3 exhibited an apparent faster on-rate ($k_{on} \sim \times 10^5$ 1/smol/L) and a somewhat faster off-rate ($k_{off} \sim 10^{-4}$ 1/s) compared to FG88.2, that could explain the slightly reduced cytotoxicity compared to FG88.2. In comparison, 88IgG1 bound its target with an apparent fast on-rate ($k_{on} \sim 10^5$ 1/smol/L), but in contrast to the mIgG3 isotypes displayed a much faster dissociation phase (apparent $k_{off} \sim 10^{-2}$ 1/smol/L), that is likely to underly its reduced cytotoxic activity upon cancer cell binding. The mAb binding behaviour on the low-density flow cell was largely comparable between the three mAbs with equilibrium dissociation constants (K_d) of the order of 10^{-8} mol/L for all three mAbs.

Example 2. Domain analysis of the mIgG3 constant region suggests a major contribution by the mIgG3 CH3 domain with a minor involvement of the CH2.

Collectively, the results suggested that the high Lewis^a-APD-HSA functional affinity exhibited by FG88 and 88mIgG3, predominantly driven by their slow dissociation, potentially resulting from the intermolecular cooperativity of this isotype, contributed to their direct cytotoxic effect on high-binding cancer cell lines. We thus set out to engineer a IgG1 cancer glycan targeting mAb with direct cytotoxic activity, via the transfer of selected mIgG3 constant region residues into 88IgG1. Firstly, mIgG3 contributing regions were identified through the creation of hybrid 88IgG1 constructs, containing mIgG3 CH1, CH2 or CH3 domains. Preliminary analyses ascertained that mIgG3 CH1 had a negligible contribution to the direct cytotoxicity ability of 88mIgG3, as introducing mIgG3 CH1 into 88IgG1 (1m1) did not lead to a significant increase in cytotoxicity (Figure 2A and 2B). Conversely, introducing IgG1 CH1 into 88mIgG3 (3h1), equally, did not instigate a significant reduction in killing activity (Figure 2A and 2B). Next, in a gain-of-function approach, the mIgG3 CH2 and CH3 domains, separately, were introduced in 88IgG1. 88IgG1 containing murine CH3 (1m3) exhibited a significant gain in PI uptake on HCT15, as well as a significant increased proliferation inhibition of COLO205 cells, when compared to 88IgG1 (Figure 2C and D). Introducing murine CH2 into 88IgG1 (1m2) led to small, but not significant, increase in killing activity across both assays (Figure 2C and D). As a confirmation of the contributions made by both domains, the reverse strategy was followed whereby a loss of cytotoxicity activity was evaluated due to the introduction IgG1 CH2 or CH3 domains into 88mIgG3. This scenario led to a significant decrease in cytotoxicity for 88mG3 containing IgG1 CH3 (3h3), corroborating the previous gain-of-function results. Importantly, this strategy also identified a small contribution by the murine CH2, as 88mIgG3 containing human CH2 (3h2) exhibited a significant decrease in cytotoxicity activity (Figure 2C and D). Next, the kinetic binding behaviour of the hybrid constructs was analysed. The hybrid construct 1m3 exhibited a modest, but significant increase in functional

affinity (decreased K_d), whilst 3h3, containing human CH3, displayed a significant decrease in functional affinity (increased K_d , Figure 2E), in both cases, mirroring the direct cytotoxicity. Human CH2 in construct 3h2 also led to a modest, but significant drop in functional affinity. In all cases, the changes in functional affinity were predominantly driven by changes in the off-rate of the mAbs, with 1m3 showing a significantly decreased off-rate compared to 88IgG1 and 3h3, as well as 3h2, exhibiting a significantly increased off-rate compared to 88mIgG3 (Figure 2F). Murine CH2 in construct 1m2 did not lead to increased functional affinity nor a decreased off-rate, underlying the insignificant cytotoxicity of this construct compared to 88IgG1 (Figure 2C and D). Taken together the results indicate that the murine CH3 has a more pronounced contribution to cytotoxicity, as well as kinetic binding, whereas the contribution by murine CH2 is smaller, only observed in a loss-of-function setting.

Example 3. Discontinuous sequences within the CH2-CH3 region of aa 286-397 are essential for killing activity and increased functional affinity.

As the cytotoxic effect endowed by the murine CH3 was not complete, and in order to further narrow down the other contributing residues, we designed hybrid 88 mAb constructs where the CH2 and CH3 domains were further subdivided into two subdomains (SD) with junction regions containing a 10 residue overlap: CH2: SD232-294 and SD286-345 and CH3: SD339-397 and SD390-447. On COLO205, both SD339-397 and SD286-345 afforded a similar significant increase in cytotoxicity, most evident at the lower concentrations, whereas SD232-294 as well as SD390-447, alone, or in combination (not shown), were dispensable for cytotoxicity (Figure 3A and C). On HCT15 however, the significant contribution by residues within SD339-397 was larger than that of SD286-345 (Figure 3B and D), suggesting that subtle differences in glyco-antigen density and composition can modulate mAb binding and ensuing cytotoxic activity. Strikingly, 88IgG1, containing the combined mIgG3 SD286-345 and SD339-397 (SD286-397), recovered virtually all the cytotoxicity of 88mIgG3 on both cell lines and across both assays (Figure 3A-D), obviating the need for adding additional subdomains. Functional affinity analysis of the subdomain constructs, compared to 88IgG1, revealed a striking improvement in functional affinity for SD286-397, as well as SD339-397, both now matching the 88mIgG3 functional affinity, with a more modest improvement for SD286-345 (Figure 3E). The improved functional affinity resulted mainly from a dramatically reduced apparent off-rate ($\sim 10^{-6}$ 1/s) for SD286-397 as well as SD339-397, with the SD286-345 off-rate showing a more modest improvement ($\sim 10^{-3}$ 1/s) (Figure 3F). These results add further weight to the cytotoxicity observations and suggest that creating a mAb with a reduced target dissociation rate supports direct cytotoxicity.

Although SD339-397, with 27 mIgG3 residues, recapitulated up to 90% of the desirable attributes of 88mIgG3, notably the slow dissociation and enhanced cytotoxicity, it exhibited a significantly reduced CDC activity compared to 88IgG1 (Figure 3G), necessitating the use of SD286-397 for further development. In contrast, ADCC activity of SD339-397 was not reduced compared to 88IgG1 (Figure 3H). Although SD286-397 fully recapitulated 88mIgG3 cytotoxicity, it still contained

41 mlgG3 residues. Consequently, additional subdivisions of SD286-345 and SD339-397 were analysed for cytotoxic activity and functional affinity. This analysis highlighted two areas, in SD339-397 and SD286-345, respectively, that contributed to direct cytotoxicity. Firstly, SD339-378, containing 20 mlgG3-specific residues, upon introduction in 88IgG1 led to a significant regain of cytotoxicity to within ~ 80% to 90% of mlgG3 cytotoxicity across both assays (Figure 4A and B). This region, also instilled a significant increase in functional affinity, compared to 88IgG1, but this improvement was not as pronounced as in the case of SD339-397 (Figure 4G). Within the same set of constructs, we identified another region of interest via the loss-of-function approach. The significant reduction in cytotoxicity by SD307-345 compared to SD286-345, implied a further contribution by residues 286 - 306 (Figure 4C). Collectively, the results suggested that a construct containing the combination of residues 286-306 and 339-378, totalling 26 mlgG3-specific residues, could potentially fully recapitulate 88mlgG3 direct cytotoxicity and functional affinity. To test this hypothesis, the cytotoxic activity and functional affinity of SD286-306+339-378 was evaluated. SD286-306+339-378 exhibited significantly improved direct cytotoxicity, compared to 88 IgG1, on both cell lines, now matching 88mlgG3 cytotoxicity (Figure 4D-F). SPR analysis of SD286-306+339-378 revealed a significantly improved functional affinity compared to 88IgG1 with a K_d (0.3×10^{-9} nmol/L) and similar to 88mlgG3 (Figure 4G). Importantly, neither the CDC activity, nor the ADCC activity of the SD286-306+339-378 construct was significantly different from that of 88IgG1 (Figure 4H and I). The combination of improved functional affinity with direct cytotoxicity, as well as maintained immune effector functions, indicates that our SD286-306+339-378 hybrid IgG1 mimics the desirable attributes of 88mlgG3.

Example 4. Reversal of one *in silico* identified immunogenic cluster generates the lead candidate, improved 'i' 88G1, with robust cell killing, pore-forming ability and sound immune effector functions.

In order to assess the potential immunogenicity of our hybrid SD286-306+339-378 construct, created by the presence of 26 mlgG3 residues, we performed an *in silico* screen of the SD286-306+339-378 sequence for MHCII-binding epitopes (Immune Epitope Database, IEDB). Class II-restricted T helper cells are more relevant to the humoral immune response and predicted binding clusters have been shown to be strong indicators of T cell responses (Jawa et al. 2013). Two MHCII binding clusters, containing several high-scoring binding epitopes, were identified: cluster 1 (residues 294-315) and cluster 2 (residues 365-393) (Figure 5/Figure 20). Reversion of three murine residues, 294 (A to E), 300 (F to Y) and 305 (A to V) back to human residues, within the cluster 1, produced a human sequence section to which individuals would have been tolerated. Similarly, reversal of two residues 351 (I to L) and 371 (N to G), within the 339-378 region, removed two MHCII binding cores. We thus created two additional SD286-306+339-378-based constructs, based on the above *in silico* screen: clusters 1 (DI1) and 2 (DI2), containing three and two human reverted residues, respectively, and assayed their cytotoxicity. DI1, as well as DI2, maintained significantly improved cytotoxicity compared to 88IgG1, but DI2 showed a small, but consistent decreased activity compared to 88mlgG3 (Figure 6A and B). Additionally, in the case of

88DI1 the direct cytotoxicity coincided with a favourable functional affinity profile, with an apparent off-rate of ($\sim 10^{-4}$ 1/s) and a K_d of 0.5×10^{-9} nmol/L that was not significantly different from 88mlgG3 (Figure 6C, Table 2). In contrast, DI2 displayed a significantly decreased functional affinity compared to 88mlgG3 (Figure 6C). Based on these finding, we focused on 88DI1, containing 23 mlgG3 residues, now renamed improved 'i' (improved) 88G1, for further analysis of its immune effector functions. 88IgG1 showed potent ADCC activity on COLO205 with sub-nanomolar EC_{50} (Figure 6D), in line with the potent immune effector functions of FG88 (Chua et al. 2015). Similarly, i88G1 displayed potent ADCC with subnanomolar EC_{50} (0.35 nmol/L) albeit somewhat reduced compared to 88IgG1 (EC_{50} 0.13 nmol/L). The CDC activity of i88G1 was modestly improved compared to 88IgG1 (Figure 6E, Table 2).

Earlier work on the parental hybridoma-produced FG88 mAb had demonstrated its pore-forming ability, which was surmised to underlie its cytotoxicity (Chua et al. 2015). We thus set out to analyse the pore-forming ability of i88G1 on HCT15, using SEM. Incubation of HCT15 with i88G1 or 88mlgG3, but not 88IgG1, resulted in monolayer disruption, cell rounding and clustering. At higher magnification, irregular pore formation was evident (Figure 6F), mirroring the original data observed for the hybridoma-produced FG88 mAb (Chua et al. 2015).

Collectively the results indicate that transfer of selected regions from the mlgG3 constant region into the 88IgG1 backbone created a hybrid mAb with direct cell killing ability, increased functional affinity as well as robust immune effector functions.

Example 5. Transfer of the 'iG1' sequences into an alternative, non-killing, glycan binding mAb (129 IgG1) creates a cancer-targeting mAb with improved *in vitro* and *in vivo* anti-tumour activity.

We recently described the generation of a sialyl-di-Lewis^a recognizing mAb (129 mAb) with development potential for cancer immunotherapy. The 129 mAb has a more favourable tumour versus normal human tissue distribution compared to the above-described 88 mAb, predominantly resulting from its very restricted normal tissue reactivity. Neither the hybridoma-produced FG129, a murine IgG1 mAb, nor the chimeric 129IgG1, exhibit direct cytotoxicity. This led us to test the hypothesis that the introduction of the 23 above-selected mlgG3 constant region residues into the Fc region of CH129 would create an 'i'129G1 with direct cytotoxicity and improved functional affinity and thus exhibit superior clinical utility.

We evaluated the direct cytotoxicity ability of i129G1 on COLO205, previously shown to be a high-binding cancer cell line for the 129 mAb. The i129G1 displayed significantly improved (compared to 129IgG1), dose-dependent proliferation inhibition (Figure 7A), with an EC_{50} of 45.6 nmol/L, as well as a significantly improved, but more modest, PI uptake (Figure 7B). Next, we analysed the functional affinity of i129G1 using a sialyl Lewis^a-APD-HSA-coated chip and SPR. The i129G1 mAb exhibited significantly improved functional affinity compared to 129IgG1 (Figure

7C, Table 2), resulting predominantly from an improvement in off-rate by almost two logs ($2.6 \times 10^{-4} \text{ s}^{-1}$ and $5.5 \times 10^{-6} \text{ s}^{-1}$ for 129IgG1 and i129G1, respectively). 129IgG1 previously exerted potent ADCC effector functions on COLO205, consequently the ADCC as well as CDC activity of i129G1 on COLO205 were evaluated. The ADCC activity of i129G1 was significantly reduced compared to that of 129IgG1, but cell lysis was maintained in the nanomolar range (EC_{50} of $2.4 \times \text{nmol/L}$ and $1.7 \times \text{nmol/L}$, for i129G and 129IgG1, respectively) (Figure 7D, Table 2). The CDC activity of i129G1, however, was significantly increased compared to the parental 129IgG1, with EC_{50} of $8.2 \times \text{nmol/L}$ and $75 \times \text{nmol/L}$, for i129G1 and 129IgG1, respectively (Figure 7E, Table 2). The direct cytotoxicity as well as improved functional affinity of i129G1 led us to analyse its pore-forming ability on COLO205. The incubation of COLO205 with i129G1, caused the formation of large cell clumps with uneven surfaces, as well as the appearance of irregular pore-like structures (Figure 7F). Incubation with 129IgG1, at the same concentration, also led to a degree of cell clumping, but smaller and fewer clumps were observed, without evidence of pore formation.

The direct cytotoxicity and improved functional affinity of i129G1 directed us towards analysing the *in vivo* anti-tumour activity of i129G1 in comparison with the parental 129IgG1 in a COLO205 xenograft model. The i129G1 mAb instigated a significant reduction in tumour volume compared to vehicle control (two-way ANOVA, $p < 0.0001$) which remained significant when compared to 129IgG1, thereby corroborating the *in vitro* results (Figure 7G). No effect on mean body weight was observed (Figure 7H).

The creation of a mAb, through the introduction of a select number of mIgG3 constant region residues, with direct cytotoxicity, improved functional affinity and superior *in vivo* anti-tumour activity paves the way to applying our strategy to other cancer-targeting mAbs with clinical utility.

Example 6. Transfer of the 'iG1' sequences into an alternative, Lewis^y glycan binding mAb (27IgG1) creates a cancer-targeting mAb with improved *in vitro* direct cell killing ability.

We also created a mono-specific Lewis^y-binding mAb, 27mAb with a wide-ranging tumour tissue distribution and favourable normal human tissue binding. This mIgG3 mAb displays direct cell killing on the Lewis^y-expressing AGS and MCF7 cell lines, whereas the chimeric 27IgG1 did not (Figure 8A and B). We introduced our selected 23 mG3 residues into 27IgG1, thereby creating i27G1 and analysed its direct cell killing. On both MCF7 and AGS, the i27G1 displays significantly improved direct cell killing, compared to 27IgG1, equivalent to the 27mG3 mAb (Figure 8A and B). Additionally, i27G1 exhibits significantly improved functional Lewis^y affinity compared to the 27IgG1 (Figure 8C). Importantly, the effector functions of i27G1, ADCC as well as CDC, were both equivalent to those of 27IgG1, on MCF7 (Figure 8D and 8E).

Collectively the results indicate that transfer of selected regions from the mIgG3 constant region into the 27IgG1 backbone created a hybrid mAb with direct cell killing ability, increased functional affinity as well as robust immune effector functions.

Example 7. Further fine-tuning of the 23 'iG1' sequences suggests that 15 or 7 residues are essential to maintain direct cell killing in a cell-type dependent manner.

In order to evaluate the individual contribution of each of the 23 selected mG3 residues, a single revertant strategy based on i88G1 (Example 4) was performed. Each of the 23 mG3 residues in i88G1, singly, were reverted to the hG1 residue and the resulting construct was analysed for direct cell killing on COLO205 as well as HCT15, using the cellular proliferation assay (CCK-8, Sigma 96992) and compared to the parental i88G1. The fixed concentration percentage killing was normalised against the corresponding percentage killing by i88G1 at the same concentration, in the same experiment, in order to relate cell killing across multiple experiments. Most i88G1 revertant constructs retained good direct cell killing against COLO205 cells (Figure 9A). Residues 342, 343, 345, 361, 362, 374 and 376 (seven in total) had a mean normalised killing less than 1 and were considered essential for the direct cell killing on the high-binding COLO205 cell line. In contrast if these seven residues alone were reverted this minimal construct did not express/fold and therefore this construct has not been pursued. Direct cell killing of the moderate-binding HCT15 proved to be more sensitive to reversal back to hG1 residues, with only eight reverted residues (339, 344, 354, 356, 357, 358, 370, 373) maintaining cell killing to within one standard deviation of i88G1, thus necessitating the retention of 15 mG3 residues: N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S (Figure 9B). The latter construct was created for both the 88 and 129mAbs, i88G1v2 and i129G1v2, respectively and their cell killing analysed on COLO205 and HCT15. Direct cell killing of COLO205 by i88G1v2 as well as i129G1v2 was equivalent to that observed by i88G1 and i129G1, respectively (Figure 9C and 9D). Direct cell killing of HCT15 by i88G1v2, equally, matched that observed by i88G1 (Figure 9E).

Collectively, the results suggest that 15 mG3 residues: N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S, suffice to induce direct cell killing on high as well as moderate-binding cancer cell lines.

Table 1. Overview of the kinetic binding parameters of the parental 88 mAbs

Real-time Lewis ^a -HSA binding ^{a,b}			
mAb	Association Rate K _{on} (1/mols/L)	Dissociation Rate K _{off} (1/s)	Dissociation Constant K _d (nmol/L)

FG88	7.0×10^4	3.6×10^{-6}	0.05
88mlgG3	5.2×10^5	1.8×10^{-4}	0.3
88lgG1	3.1×10^5	1.5×10^{-2}	48.3

^a representative of a minimum of 3 analyses

^b results for high-density surface binding are presented

5 Table 2. Overview of the functional characteristics of the improved constructs

Biological activity characteristics					
mAb	functional affinity Kd (nmol/L)	direct cytotoxicity ^a EC ₅₀ (nmol/L)	ADCC EC ₅₀ (nmol/L)	CDC EC ₅₀ (nmol/L)	Pore forming ability
88mlgG3	0.3	26.7	ND	ND	+++
88lgG1	48.3	N/A	0.13	3.9	-
i88G1	0.5	29.4	0.35	0.1	++
129lgG1	2.5	N/A	1.7	75.3	-
i129G1	0.005	45.6	2.4	8.2	++

^adeduced from proliferation inhibition on COLO205

N/A: not appropriate, ND: not determined

10 **Example 8. Generation and initial characterisation of FG27 mAbs: FG27 was raised by immunisation with gastric tumour cell glycolipid**

15 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 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.

20 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 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 10). FG27.10 and FG27.18 were both cloned. FG27.10 was an IgG3κ and
25 FG27.18 was an IgG1κ subclass. To ensure that both mAbs bound to glycolipid, glycolipid was extracted from a range of cell lines, dried 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 11).

5 **Example 9. Direct cell killing of parental FG27 mAb**

10 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.

15 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 Lewis^a mAb SC104 for 2hrs at RT, before cell death was measured by the uptake of PI, which is a DNA intercalating agent that is only taken up by dying cells (Figure 12A). 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 12B shows direct cell 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
20 showed killing of the antigen negative cell lines (LoVo, MKN45, 791T, SKOV3).

25 Figure 12C 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 modified IgG1 chimeric showed moderate titratable killing whereas the IgG2 variant did not.

Example 10. FG27 binding studies

30 The humanised (humanized 27) and chimeric mAbs (CH27) demonstrated a similar cell binding pattern on titration on cancer cell lines (Figure 13A-E). Equilibrium affinity constants are overall on the same order, but slightly higher compared to the parental murine antibodies (Figure 13F-G), 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.

35 **Example 11. Direct cell killing of the humanised FG27 mAb**

40 In order to enhance the direct cell killing of the human IgG1 27 chimeric (27IgG1), we transferred selected mIgG3 constant region residues into the IgG1 Fc domain thereby creating an improved 'i27G1' LewisY glycan binding mAb with improved *in vitro* direct cell killing ability.

Figure 14 shows direct cell killing, functional affinity as well as effector functions of the improved i27G1. The mlgG3 27 antibody has direct cell killing ability on the Lewis^y-expressing MCF7 (Figure 14i) and AGS cell lines (Figure 14ii) whereas the chimeric 27IgG1 does not. Our i27G1, containing selected mlgG3 constant region residues, displays significantly improved direct cell killing, compared to 27IgG1, equivalent to the 27mG3 mAb, on both MCF7 (Figure 14i) and AGS (Figure 14ii). Additionally, i27G1 exhibits significantly improved functional Lewis^y affinity compared to the 27IgG1 (Figure 14iii). Importantly, the effector functions of i27G1, ADCC as well as CDC, were both equivalent to those of 27IgG1, on MCF7 (Figure 14iv and 14v). Collectively the results indicate that transfer of selected regions from the mlgG3 constant region into the 27IgG1 backbone created a hybrid mAb with direct cell killing ability, increased functional affinity as well as robust immune effector functions.

Example 12. Transfer of the 'iG1' sequences into a protein binding mAb (Trastuzumab) creates a cancer-targeted mAb with improved functional affinity.

In order to evaluate if altering the amino acid residues of an antibody targeting cancer-associated proteins resulted in increased functional affinity, the 'iG1' residues (23, 'v1' as well as 15, 'v2') were altered and the affinity of the resultant, 'improved' antibody variants determined via SPR analysis. To determine the functional affinity, the three Trastuzumab antibody constructs were titrated across HER2-coated chips at three different ligand densities. Multicycle kinetic analysis (in the concentration range of 90nM – 0.37M) was performed and demonstrated increased functional affinity by iTv1 and iTv2 compared to WT on both the low density (20RU) and high density (200RU) surfaces (Figure 18A). The improvement was more pronounced on the high-density surface and was driven predominantly by a reduction in off-rate (8.5×10^{-8} 1/s for iTv1 and 7.4×10^{-8} 1/s for iTv2 versus 6.0×10^{-7} 1/s for WT). HEK293-expressed wild-type Trastuzumab (WT) and the 'improved' variants (iTv1 and iTv2) gave high binding to the HER2 expressing cell lines SKBR3 and BT474, and low to moderate binding to MDA-MB231 and SKOV3 (Figure 18B). In order to evaluate whether the more avid binding by iTv1 and iTv2 would result in differential cytotoxicity, proliferation inhibition on MDA-MB231 and PI uptake on BT474 were explored. iTv2 displayed significantly improved proliferation inhibition compared to WT on MDA-MB231 (Figure 18C), with both iTv1 and iTv2 showing improved PI uptake on BT474 cells compared to WT, most pronounced at the highest concentration tested (Figure 18D). Titration of directly HRPO-labelled mAbs onto HER2 coated ELISA plates revealed improved binding by iTv2 versus WT. Collectively the results indicate improved functional affinity by the 'iG1' engineered Trastuzumab constructs suggesting that our approach can also benefit protein-targeting mAbs, in addition to glycan targets. The final outcome of our engineering approach however, relies on the interplay between the intricate avidity of the mAb combined with the target density.

Embodiments

Certain embodiments of the present invention are described below. Various features of the following embodiments may be combined with features of other embodiments, for example structural features such as sequence motifs may be combined with functional features of the claimed antibodies or antigen binding fragments thereof.

1. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residues of an Fc-region of an immunoglobulin and a binding region, wherein one or more residues of the Fc-region are modified to the corresponding residue from a mouse IgG3 antibody and wherein the modified IgG1 antibody or antigen-binding fragment thereof has enhanced functional affinity when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
2. The modified IgG1 antibody or antigen-binding fragment thereof according to embodiment 1 wherein the functional affinity of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 10% when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
3. The modified IgG1 antibody or antigen-binding fragment thereof according to embodiment 1 or embodiment 2 wherein the functional affinity of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 100% when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
4. The modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1 to 3, wherein the modified IgG1 antibody or antigen-binding fragment thereof has enhanced direct cell-killing when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
5. The modified IgG1 antibody or antigen-binding fragment thereof according to embodiment 4 wherein the direct cell-killing of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 10% when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
6. The modified IgG1 antibody or antigen-binding fragment thereof according to embodiment 1 or embodiment 2 wherein the direct cell-killing of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 20%, at least about 30%, at least about 40%, at

least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 100% when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.

5 7. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more residues of the Fc-region are selected from: **Q342, P343, E345, N361, Q362, P374, D376.**

10 8. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more modified residues of the Fc-region are selected from: **Q342R, P343A, E345T, N361K, Q362K, P374S, D376A.**

15 9. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more residues of the Fc-region are selected from: **N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378.**

20 10. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more modified residues of the Fc-region are selected from: **N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S.**

25 11. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more residues of the Fc-region are selected from: **N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.**

30 12. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more modified residues of the Fc-region are selected from: **N286T, K288W, K290Q, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.**

35 13. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more residues of the Fc-region are selected from: **N286, K288, K290, E294, Y300, V305, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.**

14. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the one or more modified residues of the Fc-region are selected from: **N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I,**

S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.

15. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the antibody or antigen-binding fragment thereof is a human antibody, humanised antibody or chimeric antibody.

16. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding embodiment, wherein the IgG1 antibody or antigen-binding fragment thereof is a monospecific antibody, bispecific antibody or multispecific antibody.

17. The modified IgG1 antibody or antigen-binding fragment thereof according to embodiment 16, wherein the IgG1 antibody or antigen-binding fragment thereof is a bispecific antibody comprising an Fc-region with a first heavy chain and a first antigen-binding region, a second heavy chain and a second antigen-binding region.

18. The modified IgG1 antibody or antigen-binding fragment thereof according to embodiment 16 or 17, wherein the IgG1 antibody or antigen-binding fragment thereof is a bispecific antibody that binds to a CD3 antigen.

19. A modified IgG1 antibody or antigen-binding fragment thereof comprising the motif RAXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXSA, wherein X is any amino acid.

20. A modified IgG1 antibody or antigen-binding fragment thereof comprising the motif TXWXQXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXRAXXXXXX IXXXXXXXXSXKKXXXXXXXXNXXSEAXS, wherein X is any amino acid.

21. A modified IgG1 antibody or antigen-binding fragment thereof comprising the motif TXWXQXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXPXXRAQTXXXX XIXXPXEQMSXKKXXXXXXXXTXFSEAXS, wherein X is any amino acid.

22. A modified IgG1 antibody or antigen-binding fragment thereof comprising the motif TXWXQXXXAXXXXXFXXXXAXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXPXXRAQTXXXX XIXXPXEQMSXKKXXXXXXXXTNXFSEAXS, wherein X is any amino acid.

23. A modified IgG1 antibody or antigen-binding fragment thereof comprising any one or more motifs selected from the following list:

(i) TXWXQ

(ii) RAXXXXXXI

(iii) SXKKXXXXXXXXNXXSEAXS

(iv) NXXSEAXS

wherein X is any amino acid.

24. A method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof comprising one or more residues of an Fc-region of an immunoglobulin and a binding region, wherein the method comprises replacing one or more residues of the CH2 and/or the CH3 domains of said Fc-region with one or more corresponding residues from mouse IgG3 CH2 and/or CH3 domains.
25. The method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof according to embodiment 24 wherein the method comprises modifying one or more residues of the Fc region selected from: **Q342, P343, E345, N361, Q362, P374, D376.**
26. The method according to embodiment 25 wherein the one or more modified residues are selected from: **Q342R, P343A, E345T, N361K, Q362K, P374S, D376A.**
27. The method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof according to embodiment 24 wherein the method comprises modifying one or more residues of the Fc region selected from: **N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378.**
28. The method according to embodiment 27 wherein the one or more modified residues are selected from: **N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S.**
29. The method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof according to embodiment 24 wherein the method comprises modifying one or more residues of the Fc region selected from: **N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.**
30. The method according to embodiment 29 wherein the one or more modified residues are selected from: **N286T, K288W, K290Q, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.**
31. The method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof according to embodiment 24 wherein the method comprises modifying one or more residues of the Fc region selected from: **N286, K288, K290, E294, Y300, V305, A339,**

Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.

5 32. The method according to embodiment 31 wherein the one or more modified residues are selected from: **N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.**

10 33. A pharmaceutical composition comprising the modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1 to 23, and a pharmaceutically acceptable carrier.

15 34. The pharmaceutical composition according to embodiment 33, further comprising at least one other therapeutic agent.

35 35. The pharmaceutical composition according to embodiment 33 or 34 comprising the modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1 to 23 and an immunomodulatory agent.

20 36. A modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1 to 23 or a composition according to any one of embodiments 33-35 for use as a medicament.

25 37. A modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1 to 23 or a composition according to any one of embodiments 33-35 for use in the treatment of cancer, autoimmune diseases, inflammatory diseases or infectious diseases.

30 38. A method of treating an individual having a disease comprising administering to said individual a pharmaceutically effective amount of a modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1 to 23 or a composition according to any one of embodiments 33-35.

35 39. The method of treating an individual according to embodiment 38, wherein the disease is selected from the group of cancer, autoimmune disease, inflammatory disease and infectious disease.

40 40. The method of treating an individual according to any one of 38-39, wherein the method further comprises administering an additional therapeutic agent to the individual.

41. The method of treating an individual according to embodiment 40 wherein the additional therapeutic agent is an immunomodulatory agent.

42. A kit comprising a modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1-23 or a composition according to any one of embodiments 33-35, wherein said modified IgG1 antibody or antigen-binding fragment thereof, or composition is in one or more containers such as vials.
43. The kit according to embodiment 42, wherein the modified IgG1 antibody or antigen-binding fragment thereof or composition is for simultaneous, separate or sequential use in therapy.
44. Use of a modified IgG1 antibody or antigen-binding fragment thereof according to any one of embodiments 1-23 or a composition according to any one of embodiments 33-35 for the manufacture of a medicament for treatment of a disease.
45. The use according to embodiment 44, wherein the disease is cancer, autoimmune disease, inflammatory disease or infectious disease.
46. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residue modifications to the Fc region at residue positions selected from: **Q342, P343, E345, N361, Q362, P374, D376**.
47. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more modifications to the Fc region selected from: **Q342R, P343A, E345T, N361K, Q362K, P374S, D376A**.
48. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residue modifications to the Fc region at residue positions selected from: **N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378**.
49. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more modifications to the Fc region selected from: **N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S**.
50. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residue modifications to the Fc region at residue positions selected from: **N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378**.
51. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more modifications to the Fc region selected from: **N286T, K288W, K290Q, A339P, Q342R, P343A,**

R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.

52. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residue
5 modifications to the Fc region at residue positions selected from: **N286, K288, K290, E294, Y300, V305, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.**

53. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more
10 modifications to the Fc region selected from: **N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.**

54. A modified IgG1 antibody or antigen binding fragment thereof comprising one or more of the
15 sequences disclosed in Figure 19.

55. A modified IgG1 antibody or antigen binding fragment thereof comprising a heavy chain and a
light chain sequence as disclosed in Figure 19.

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Claims

1. A modified IgG1 antibody or antigen-binding fragment thereof comprising one or more residues of an Fc-region of an immunoglobulin and a binding region, wherein one or more residues of the Fc-region are modified to the corresponding residue from a mouse IgG3 antibody and wherein the modified IgG1 antibody or antigen-binding fragment thereof has enhanced functional affinity when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
2. The modified IgG1 antibody or antigen-binding fragment thereof according to claim 1 wherein the functional affinity of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 10% when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
3. The modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1 to 2, wherein the modified IgG1 antibody or antigen-binding fragment thereof has enhanced direct cell-killing when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
4. The modified IgG1 antibody or antigen-binding fragment thereof according to claim 3 wherein the direct cell-killing of the modified IgG1 antibody or antigen-binding fragment thereof is enhanced by at least about 10% when compared to a corresponding IgG1 antibody or antigen-binding fragment thereof comprising wildtype Fc-region residues.
5. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more residues of the Fc-region are selected from: **Q342, P343, E345, N361, Q362, P374, D376**.
6. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more modified residues of the Fc-region are selected from: **Q342R, P343A, E345T, N361K, Q362K, P374S, D376A**.
7. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more residues of the Fc-region are selected from: **N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378**.
8. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more modified residues of the Fc-region are selected from: **N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S**.

9. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more residues of the Fc-region are selected from: **N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.**
10. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more modified residues of the Fc-region are selected from: **N286T, K288W, K290Q, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.**
11. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more residues of the Fc-region are selected from: **N286, K288, K290, E294, Y300, V305, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378.**
12. The modified IgG1 antibody or antigen-binding fragment thereof according to any preceding claim, wherein the one or more modified residues of the Fc-region are selected from: **N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.**
13. A method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof comprising one or more residues of an Fc-region of an immunoglobulin and a binding region, wherein the method comprises replacing one or more residues of the CH2 and/or the CH3 domains of said Fc-region with one or more corresponding residues from mouse IgG3 CH2 and/or CH3 domains.
14. The method of increasing the direct cell killing ability of an IgG1 antibody or antigen-binding fragment thereof according to claim 13 wherein the method comprises modifying one or more residues of the Fc region selected from:
- (a) **Q342, P343, E345, N361, Q362, P374, D376**, optionally wherein the one or more modified residues are selected from: **Q342R, P343A, E345T, N361K, Q362K, P374S, D376A**;
 - (b) **N286, K288, K290, Q342, P343, E345, L351, T359, N361, Q362, G371, P374, S375, D376, A378**, optionally wherein the one or more modified residues are selected from: **N286T, K288W, K290Q, Q342R, P343A, E345T, L351I, T359S, N361K, Q362K, G371N, P374S, S375E, D376A, A378S**;
 - (c) **N286, K288, K290, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378**, optionally wherein the one or more modified residues are selected from: **N286T, K288W, K290Q, A339P, Q342R, P343A,**

R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S; or

- 5 (d) N286, K288, K290, E294, Y300, V305, A339, Q342, P343, R344, E345, L351, S354, D356, E357, L358, T359, N361, Q362, K370, G371, Y373, P374, S375, D376, A378, optionally wherein the one or more modified residues are selected from: N286T, K288W, K290Q, E294A, Y300F, V305A, A339P, Q342R, P343A, R344Q, E345T, L351I, S354P, D356E, E357Q, L358M, T359S, N361K, Q362K, K370T, G371N, Y373F, P374S, S375E, D376A, A378S.

10 15. A pharmaceutical composition comprising the modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1 to 12, and a pharmaceutically acceptable carrier.

15 16. The pharmaceutical composition according to claim 15, further comprising at least one other therapeutic agent, optionally wherein the additional agent is an immunomodulatory agent.

17. A modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1 to 12 or a composition according to any one of claims 15 or 16 for use as a medicament.

20 18. A modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1 to 12 or a composition according to any one of claims 15 or 16 for use in the treatment of cancer, autoimmune diseases, inflammatory diseases or infectious diseases.

25 19. A method of treating an individual having a disease comprising administering to said individual a pharmaceutically effective amount of a modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1 to 12 or a composition according to any one of claims 15 or 16.

30 20. The method of treating an individual according to claim 19, wherein the disease is selected from the group of cancer, autoimmune disease, inflammatory disease and infectious disease.

21. The method of treating an individual according to any one of 19-20, wherein the method further comprises administering an additional therapeutic agent to the individual, optionally wherein the additional therapeutic agent is an immunomodulatory agent.

35 22. A kit comprising a modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1-12 or a composition according to any one of claims 15-16, wherein said modified IgG1 antibody or antigen-binding fragment thereof, or composition is in one or more containers such as vials.

40 23. The kit according to claim 22, wherein the modified IgG1 antibody or antigen-binding fragment thereof or composition is for simultaneous, separate or sequential use in therapy.

24. Use of a modified IgG1 antibody or antigen-binding fragment thereof according to any one of claims 1-12 or a composition according to any one of claims 15-16 for the manufacture of a medicament for treatment of a disease.

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25. The use according to claim 24, wherein the disease is cancer, autoimmune disease, inflammatory disease or infectious disease.