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Summary: During last two decades, the chimerization and humanization of monoclonal antibodies (mAbs) have led to the approval of several for the treatment of cancer, autoimmune diseases, and transplant rejection. Additional approaches have been used to further improve their *in vivo* activity. These include combining them with other modalities such as chemotherapy and redesigning them for improved pharmacokinetics, effector function, and signaling activity. The latter has taken advantage of new insights emerging from an increased understanding of the cellular and molecular mechanisms that are involved in the interaction of immunoglobulin G with Fc receptors and complement as well as the negative signaling resulting from the hypercrosslinking of their target antigens. Hence, mAbs have been redesigned to include mutations in their Fc portions, thereby endowing them with enhanced or decreased effector functions and more desirable pharmacokinetic properties. Their valency has been increased to decrease their dissociation rate from cells and enhance their ability to induce apoptosis and cell cycle arrest. In this review we discuss these redesigned mAbs and current data concerning their evaluation both *in vitro* and *in vivo*.

Keywords: monoclonal antibody, Fc, effector function, pharmacokinetics, tumors

Introduction

Since the early 1980s, monoclonal antibodies (mAbs) have made the transition from discovery to clinical practice. Several of these mAbs, alone, or attached to payloads, are being used for the treatment of cancer. The first mAb approved by the Food and Drug Administration (FDA) for human use was a murine anti-CD3 mAb, muromonab (OKT3), used for the treatment of organ transplant rejection (1). OKT3 provided the first lesson in the mAbs field, i.e. murine mAbs had a short half-life, robust immunogenicity, and suboptimal effector functions when used in humans. These problems were solved by converting mouse mAbs into human protein formats by genetic engineering. Between 1984 and 1988, both chimerization and humanization of mAbs were achieved (2–4). A chimeric mAb is designed to contain the variable regions of a mouse antibody and the constant regions of a human antibody, while in a humanized mAb, only the complementary determining regions (CDRs) are of murine origin. Fully human mAbs were

created either by phage display or by the selection of variable regions of human origin (5). In addition, human immunoglobulin (Ig) transgenic mice are now used to produce fully human antibodies (6). The first mAb approved for the treatment of cancer, rituximab (RituxanTM), a chimeric anti-human CD20 mAb, was approved in 1997. A decade later, eight other mAbs have been approved for the treatment of cancer, and hundreds are undergoing preclinical and clinical evaluation.

FDA approved mAbs for the treatment of cancer (Table 1)

Rituximab is a chimeric IgG1 anti-CD20 mAb that binds to human B lymphocytes and has been approved for treating B-cell malignancies such as non-Hodgkin's lymphoma (NHL) either as monotherapy or in combination with chemotherapy (7). The *in vivo* mechanisms of action of rituximab include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), growth inhibition, Fas-induced apoptosis, and chemo- and radiosensitization of tumor cells (8, 9).

Trastuzumab (HerceptinTM) is a humanized IgG1 designed to bind to the extracellular domain of the human Her-2/neu receptor, a member of epidermal growth factor receptor (EGFR) family (10). It was approved by the FDA in 1998 for the treatment of metastatic Her-2-overexpressing breast cancer. It is being further evaluated as adjuvant therapy for early breast cancers. The mechanisms of action of trastuzumab are not completely understood. Trastuzumab blocks the shedding of the extracellular domain of Her-2 and inhibits metalloproteinase activity in Her-2-overexpressing breast cancer cell lines such as SKBR3 and BT474. It also interferes with signaling via phosphoinositide 3-kinase (PI3K) pathways, thus promoting apoptosis and cell cycle arrest (CCA) during the G1 phase of the cell cycle (11). Trastuzumab induces the expression of anti-angiogenic factors (e.g. thrombospondin-1) and suppresses pro-angiogenic factors [e.g. transforming growth factor- α (TGF- α), vascular endothelial growth factor (VEGF), angiopoietin-1, and plasminogen-activator inhibitor-1] (12). Trastuzumab can also mediate ADCC (13).

Alemtuzumab (Campath-1HTM) is a humanized IgG1 anti-CD52 mAb that was approved in 2001 by the FDA for the treatment of drug-resistant chronic lymphocytic leukemia (CLL) (14). CD52 is a cellular surface glycoprotein expressed by both normal and malignant B and T lymphocytes, natural killer (NK) cells, monocytes, macrophages, eosinophils, thymocytes, and spermatozoa. Campath-1M, the parental mAb of alemtuzumab, is a rat IgM used for T-cell depletion in the prophylaxis of graft-versus-host disease (14).

Alemtuzumab is the humanized variant of Campath-1G, a rat IgG2b, generated by grafting the CDRs of Campath-1G onto a human IgG1 framework (15). This mAb acts *in vitro* by CDC and ADCC and also induces apoptosis (16, 17). The *in vivo* mechanisms of action of Alemtuzumab remain unclear.

Cetuximab (ErbixTM) is a chimeric IgG1 directed against Her-1, a member of EGFR family (18). Cetuximab was approved by the FDA in 2004 for the treatment of colorectal carcinoma and is being evaluated in patients with head and neck tumors that overexpress EGFR. Cetuximab inhibits the proliferation of Her-1-expressing cells by inhibiting mitogen-activating protein kinase (MAPK)/PI3K/Akt signaling pathways, upregulating p27KIP1 expression, and inducing apoptosis. It also can inhibit the production of VEGF and mediate ADCC and CDC (18–20).

Bevacizumab (AvastinTM) is a humanized anti-angiogenic mAb (IgG1) that targets VEGF (21, 22) but is rarely cytotoxic for tumor cells themselves (23). In 2004 the FDA approved bevacizumab in combination with 5-fluorouracil as first line treatment for metastatic colorectal cancer.

Panitumumab (VectibixTM) is a human IgG2 directed against EGFR (24, 25). In 2006, panitumumab was approved by the FDA for the treatment of metastatic colorectal cancer. Panitumumab acts on cancer cells by inhibiting growth, by inducing apoptosis, and by decreasing of the production of pro-inflammatory cytokines and VEGF (24, 25).

Gemtuzumab ozogamicin (MylotargTM) is a humanized IgG4 anti-CD33 conjugated to calicheamicin, a cytotoxic antibiotic (26). CD33 is a glycoprotein receptor expressed on normal and monomyeloid hematopoietic progenitor cells (27). In 2000 gemtuzumab ozogamicin was approved by the FDA for the treatment of patients with acute myeloid leukemia (AML). After specific binding to the target cell, the ligand–receptor complex is internalized, and the drug is hydrolyzed and released inside the cell where it causes double-strand DNA breaks, and consequently apoptosis. Presently, there are ongoing clinical studies in patients with AML using gemtuzumab ozogamicin in combination with chemotherapy.

Ibritumomab tiuxetan (ZevalinTM) and tositumomab (BexxarTM) are two radioimmunoconjugates directed against CD20. Ibritumomab tiuxetan is a murine IgG1 conjugated to the radioisotope 90-yttrium (28, 29). In 2002, ibritumomab tiuxetan was approved by the FDA for the treatment of refractory low grade and follicular B-cell NHLs. In 2003, the second radioimmunoconjugate, tositumomab, was approved by the FDA for the treatment of relapsed and refractory follicular NHL after chemotherapy. Tositumomab is a murine

Table 1. Monoclonal antibodies approved by the FDA for cancer therapy

Generic (Trade mark)	Target antigen	Isotype	Species	Payload	Mechanism of action	Antitumor therapeutic activity	FDA year of approving	Reference
Rituximab (Rituxan TM)	CD20	IgG1 k	Chimeric	–	Induction of apoptosis, ADCC, CDC, chemosensitization	Low grade B-cell NHL	1997	(7–9, 45, 46)
Trastuzumab (Herceptin TM)	Her-2/neu	IgG1 k	Humanized	–	ADCC, chemosensitization, CCA, inhibition of angiogenesis	Her-2 overexpressed metastatic breast cancer	1998	(10–13, 82)
Alemtuzumab (Campath-1H TM)	CD52	IgG1 k	Humanized	–	ADCC, CDC	B-cell CLL	2001	(14–17)
Cetuximab (Erbix TM)	EGFR (Her-1)	IgG1 k	Chimeric	–	Inhibition of angiogenesis, chemosensitization and radiosensitization, CCA, ADCC	Metastatic colorectal cancer, head and neck cancers	2004	(18–20)
Bevacizumab (Avastin TM)	VEGF	IgG1 k	Humanized	–	Inhibition of angiogenesis	Colorectal cancer	2004	(21–23, 37)
Panitumumab (Vectibix TM)	EGFR	IgG2 k	Human	–	Inhibition of cell growth, induction of apoptosis, decreased proinflammatory cytokines and VEGF production	Metastatic colorectal cancer	2006	(24, 25)
Gemtuzumab ozogamicin (Mylotarg TM)	CD33	IgG4 k	Humanized	Calicheamicin	Double-stranded DNA breaks and cellular death induced by payload after intracellular hydrolysis	CD33 ⁺ relapsed AML	2000	(26, 27)
Ibritumomab tiuxetan (Zevalin TM)	CD20	IgG1 k	Murine	90-yttrium	Cellular death induced by β -emitter, induction of apoptosis, ADCC, CDC	Low grade or follicular, relapsed or refractory, CD20 ⁺ B-cell NHLs; Rituximab-refractory follicular NHL	2002	(28, 29)
Tositumomab (Bexxar TM)	CD20	IgG2a λ	Murine	131-iodine	Cellular death induced by γ -emitter, induction of apoptosis, ADCC, CDC	Relapsed CD20 ⁺ B-cell NHL; Rituximab-refractory NHL	2003	(29–32)

FDA, Food and Drug Administration; IgG, immunoglobulin G; ADCC, antibody-dependent cell cytotoxicity; CDC, complement dependent cytotoxicity; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; Her-2, human-2 epidermal growth factor receptor-2; CCA, cell cycle arrest; EGFR, epidermal growth factor receptor; Her-1, human-1 epidermal growth factor receptor-1; VEGF, vascular endothelial growth factor; AML, acute myeloid leukaemia.

IgG2a radiolabeled with 131-iodine (29–32). The rationale for choosing mouse mAbs for radioimmunoconjugation is that their half-life in humans is short due to their very low affinity for the human Fc neonatal receptor (FcRn) (33, 34). This short half-life reduces the systemic myeloablative side effects of the radioisotope.

There are numerous other mAbs being tested in clinical trials (Table 2). While problems associated with immunogenicity, poor effector functions, and pharmacokinetics have been

solved to a large degree by chimerization and humanization, mAb therapy is still in need of further improvements. Strategies to enhance anti-tumor activity and to improve the clinical responses induced by mAbs are therefore ongoing, and these include the evaluation of different dose regimens, disease indications, combination therapies and finally, molecules with improved functional features (35). In this review, we focus on the optimization of mAb structure.

Table 2. Monoclonal antibodies currently undergoing human clinical testing for cancer therapy in the USA

Name	Target antigen	Isotype	Specie	Payload	Antitumor therapeutic activity	Clinical trial phase	Reference
WX-G250 (Rencarex™) Ipilimumab (MDX-010)	Carbonic anhydrase IX CTLA-4	IgG1 IgG1 k	Chimeric Human	–	RCC stages I–III Malignant melanoma stages I–III Metastatic pancreatic cancer	III III II	(174, 175) (176, 177)
Zanolimumab (HuMax-CD4) Ofatumumab (HuMax-CD20)	CD4 CD20	IgG1 k IgG1	Human Human	–	Malignant melanoma stages I–III, CTCL Drug resistant B-cell CLL, low grade or follicular; relapsed or refractory, CD20+ B-cell NHLs; Rituximab-refractory follicular NHL	III III	(178, 179) (180)
ch14.18 Zalutumumab (HuMax-EGFr)	Ganglioside GD2 EGFR	IgG IgG1 k	Chimeric Human	–	Neuroblastoma Head and neck cancer (squamous cell type), NSCLC stage III	III III	(181) (182, 183)
Oregovomab (B43.1.3, OvaRex™)	CA 125	IgG1	Murine	–	Advanced varian cancer (epithelial adenocarcinoma type)	III	(184, 185)
Edrecolomab (IGN-101, Panorex™)	I7-1A (Ep-CAM)	IgG2a	Murine	–	Colorectal cancer	III	(186)
¹³¹ I-chTNT-1/B (Cotara™) Pemtumomab (R-1549, Theragyn™) Lintuzumab (SGN-33) Labetuzumab (hMN14, CEAcide™) Catumaxomab (Removab™)	DNA/histone H1 complex MUC1 CD33 CEA Ep-CAM and CD3	IgG IgG1 IgG1 IgG Trifunctional bispecific IgG1 k	Human Murine Humanized Murine Rat x mouse quadroma Chimeric	I31-iodine 90-ytrium – – –	Solid tumors Ovarian cancer AML Colorectal cancer Gastric adenocarcinoma, advanced ovarian cancer, malignant ascites Homone refractory prostate cancer; relapsed or refractory multiple myeloma, advanced RCC	III III III III II II	(187) (188) (189) (190) (191, 192) (193, 194)
CNTO 328 (cCLB8)	IL-6	IgG1 k	Chimeric	–	–	II	–
3F8 I77Lu- j591	Ganglioside GD2 PSMA	IgG3 IgG	Murine Murine	I31-iodine I77-lutetium	CNS or leptomeningeal neoplasms Metastatic androgen-independent prostate cancer	II/III II	(195) (196)
Nimotuzumab	EGFR (Her-1)	IgG1 k	Humanized	–	Metastatic colorectal cancer, lung cancer; metastatic pancreas cancer	II	(197, 198)
SGN-30	CD30	IgG1	Chimeric	–	Relapsed or refractory Hodgkin's lymphoma, anaplastic large cell lymphoma	II	(199, 200)
Ticilimumab (CP-675206) Dacizumab (Zenapax™) Epratuzumab (hLL2, LymphoCide™) 80Y-Epratuzumab Galiximab (IDEC-114) MDX-060	CTLA-4 CD25 CD22 CD22 CD80 CD30	IgG2 IgG1 IgG1 IgG1 IgG1 λ IgG1 k	Human Humanized Humanized Humanized Chimeric Human	– – – 90-ytrium – –	Metastatic melanoma Adult T-cell leukemia/lymphoma Relapsed CD22+ ALL, NHL NHL Relapsed or refractory NHLs Relapsed or refractory Hodgkin's lymphoma	II II II II II II	(201) (202, 203) (80, 204, 205) (206) (207) (208)
CT-011 CS-1008 SGN-40 Mapatumumab (TRM-1)	B7 DR5 CD40 TRAIL-R1	IgG IgG IgG IgG1	Humanized Humanized Humanized Human	– – – –	Diffuse large B-cell lymphoma Metastatic pancreatic cancer Diffuse large B-cell lymphoma Relapsed or refractory multiple myeloma, NHL, colorectal cancer; NSCLC	II II II II	(209) (210) (211, 212) (213)
Apolizumab (Hu1D10, Remitogen™) Volociximab (M200)	HLA DRB α5β1 integrin	IgG1 IgG4	Humanized Chimeric	– –	B-cell NHL, refractory CLL Advanced ovarian cancer	II II	(205, 214) (215, 216)

mAb, monoclonal antibody; IgG, immunoglobulin G; RCC, renal cell cancer; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; CTLA-4, cytotoxic T lymphocyte antigen-4; CTCL, cutaneous T-cell lymphoma; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; Ep-CAM, epithelial cell adhesion molecule; MUC1, mucin receptor; CEA, carcinoembryonic antigen; IL-6, interleukin-6; CNS, central nervous system; PSMA, prostate-specific membrane antigen; Her-1, epidermal growth factor receptor; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; DR5, death receptor; TRAIL-R1, tumor necrosis factor apoptosis-inducing ligand receptor 1; HLA DRB, human leukocyte antigen.

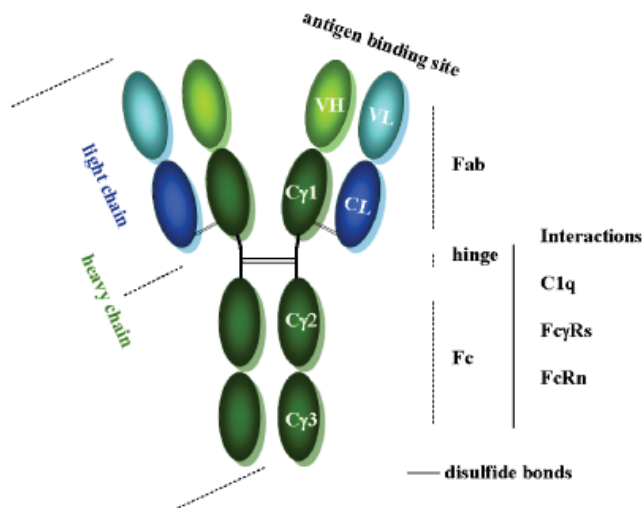


Fig. 1. The structure of IgG. VH, heavy chain variable domain; VL, light chain variable domain; Cγ1, Cγ2, and Cγ3, heavy chain constant domains; CL, light chain constant domain; Fab, antigen-binding fragment; Fc, constant region fragment; C1q, first component of complement; FcγRs, Fcγ receptors; FcRn, neonatal Fc receptor. Each heavy chain and light chain contains a variable region and a constant region. The antigen-binding sites comprise the variable regions of the heavy and light chains. The heavy constant regions (Cγ2, and Cγ3) and the hinge interact with C1q and Fc receptors and are responsible for the effector functions and pharmacokinetics of the IgG.

The structure of IgG: mechanism of action

Among different human Ig isotypes, IgG was chosen for cancer therapy due to its acceptable molecular weight (MW), *in vivo* stability, excellent effector functions, and the relative ease of producing it. The intact molecule is composed of four polypeptide chains, including two identical light chains and two identical heavy chains. The chains are joined by disulfide bonds. Human IgG subclasses include IgGs 1, 2, 3, and 4, with either κ or λ light chains within any single antibody molecule. Each heavy and light chain contains a variable region and a constant region (Fig. 1). The variable regions determine the specificity, diversity, and affinity of antigen binding, while the constant regions are responsible for mediating effector functions and controlling their catabolism. The specificity of the variable regions is a result of recombination of variable (V), diversity (D), and junctional (J) gene segments of the heavy chain and the VJ gene segments of the light chain. These genes are rearranged during B-cell maturation to generate the DNA that encodes the unique antibody combining site. The hypervariable regions or CDRs of IgG are also responsible for the strength of binding (affinity) of the antibody. Divalent antigen-binding fragments $F(ab')_2$ of some mAbs can signal target cells to undergo CCA or apoptosis.

The heavy constant regions (CH₂ and CH₃) of the IgG molecule are responsible for its effector functions, because they express binding sites for complement and for Fc receptors (FcRs) on different effector cells. The interaction of an antibody with complement initiates CDC, and FcγR interactions mediate ADCC and antibody-dependent cell phagocytosis (ADCP). FcRn-binding sites also control the catabolism of IgG.

The anti-tumor activity of IgG

There are two major mechanisms involved in the anti-tumor activity of an IgG, and these are mediated by its Fab and Fc portions.

Fab-mediated activities

Neutralization of cytokines and angiogenic factors

There are numerous immunosuppressive and angiogenic cytokines secreted by cancer cells including TGF-β and VEGF. It has been demonstrated that an anti-TGF-β mAb suppresses intra-abdominal tumors and lung metastases or transiently inhibits the growth of established breast carcinomas in mice (36). Bevacuzumab, a humanized IgG1, binds to VEGF-A with high affinity and neutralizes all human VEGF-A isoforms and their bioactive proteolytic fragments, thus inhibiting angiogenesis in tumor sites (37).

Signaling

Signaling by mAbs was first demonstrated using mAb anti-Fas (CD95) that induced apoptosis in CD95⁺ cells (38). Many tumor-associated antigens belong to the growth factor receptor family, and under normal circumstances, their activation initiates and maintains cellular growth and survival. mAbs can either induce CCA and/or apoptosis or inhibit the function of the targeted receptor [e.g. anti-VEGF receptor inhibits angiogenesis, and type 1 insulin-like growth factor receptor (IGF-1R) blocks the IGF signaling pathway] after binding to specific receptors on target cells (9, 39–44). Hence, mAbs can interfere with tumor cell growth, induce apoptosis, and, in some cases, sensitize cells to chemotherapeutic agents.

The chimeric anti-Her-1 mAb, cetuximab, blocks EGFR signaling (19). The binding of cetuximab to Her-1 induces internalization of the antibody–receptor complex without activating tyrosine kinases. Consequently, inhibition of downstream processes prevents cell growth and survival. Trastuzumab induces negative signaling in breast cancer cells that overexpress Her-2 (11).

Direct induction of apoptosis can be induced by anti-CD20 mAbs such as rituximab. Cross-linking of anti-CD20 in B cells

also leads to the translocation of CD20 into lipid rafts, followed by activation of the Src kinase family members, an elevation in intracellular Ca^{2+} , and phospholipase $\text{C}\gamma$ activation. Cross-linking results in rapid upregulation of Bax, changes in RNA level of c-myc and Berg36, activation of MAPK family members, and increased activator protein-1 (AP-1) DNA binding activity (9).

Other anti-apoptotic signaling pathways, including the extracellular signal regulated kinases 1 and 2 (ERK1/2) and the nuclear factor κB (NF- κB) pathway, have been reported to be inhibited by rituximab, resulting in the chemosensitization of various B cell lines to chemotherapy (45, 46).

Drug delivery

mAbs can be designed to carry payloads (such as chemical drugs, toxins, radionuclides, enzymes, or cytokines) to cancer cells. This delivery leads to cell death with a reduction but not complete elimination of undesirable side effects.

Fc-mediated activities

Recruitment of effector cells via Fc-Fc γ R interactions. IgGs interact with FcR-expressing effector cells through their Fc domain. Activating Fc γ Rs mediate ADCC and ADCP, induce endocytosis of immune complexes leading to antigen presentation, and contribute to the production and release of cytokines and proinflammatory molecules.

Mice deficient in Fc γ Rs (I and III) exhibit impaired ADCC and phagocytosis of IgG1-opsonized particles (47). Anti-tumor activity is dramatically reduced in common Fc γ -deficient athymic mice (lacking activating Fc γ Rs) treated with Rituximab for human lymphoma or treated with Trastuzumab for human breast cancer (48). Furthermore, a mutation in the Fc domain of an anti-Her-1 mAb (Asp265Ala) designed to abolish Fc-Fc γ RIIA interactions leads to impaired ADCC (48). In addition, mAbs have enhanced ADCC activity in mice deficient for inhibitory Fc γ RIIB receptors. Thus, Trastuzumab used at a 10-fold lower dose than that currently used therapeutically, protected the Fc γ RIIB-deficient mice xenografted with human breast cancer cells (48).

Binding of C1q

The classical activation pathway of CDC is triggered when C1, the first component of the pathway, binds to the IgG in an antigen-antibody complex. Subsequent activation of the complement cascades eventually induces the formation of a C5-C9 membrane attack complex that leads to the death of the target cell. Rituximab does not exert any anti-tumor activity in C1q-deficient mice xenografted with human CD20-transfected T

thymoma cells (49), and, in addition, depletion of complement by cobra venom factor dramatically decreases the activity of rituximab in severe combined immunodeficient (SCID) mice xenografted with human B-cell lymphomas (50). These experiments have clearly demonstrated that CDC is critical for the therapeutic activity of rituximab in mice xenografted with human tumor cells.

Engineering IgG molecules to increase their anti-tumor activity

The tumoricidal mechanisms mediated by mAbs are incompletely understood. mAbs act in a very complex manner involving both effector functions and events involved in their binding to target antigens, i.e. growth inhibition, apoptosis, etc. The contribution of each activity to the anti-tumor effect of a mAb depends upon the tumor, the target antigen, and the mAb. The future holds many challenges for designing the ideal mAbs for each tumor or group of tumors. Thus, there are many potential approaches for increasing the anti-tumor activity of mAbs, but the success of these approaches requires understanding the structure and function of the antibody molecule vis-à-vis delivering a lethal hit to tumor cells.

Specificity

A targeted therapeutic mAb must bind very specifically to tumor cells without any cross reaction with life-sustaining normal cells. The only known tumor-specific antigen is the idiotype (Id) on the antigen-specific receptor expressed on B or T cells (51, 52). Results of studies using anti-Id antibody as passive immunotherapy for treating clonal B-cell lymphoma were encouraging, but specific anti-Ids had to be prepared for each patient. Furthermore, Id⁺ tumor cells grew out, because mAbs only target one epitope on the Id (53). Active immunotherapy for B-cell lymphoma is ongoing using either natural or recombinant Ids or Id-encoding DNA vaccines (54). The results are encouraging, but costs are a major limitation in developing Id-based strategies to ablate tumors.

Affinity

It was initially assumed that increasing the affinity of mAbs should lead to increased anti-tumor activity. While this is true, it does have its limitations. mAbs with affinities above 10^{10} M^{-1} do not have improved anti-tumor activity, due to the fact that tumor penetration and retention are impaired (55). Indeed, decreasing the affinity of mAbs for some receptors can actually increase anti-tumor activity. In addition, reducing the affinity of a mAb for Fc γ RIIB increases anti-tumor

efficacy (48). Finally, mutants with low affinities for the FcRn are used for designing immunoconjugates carrying cytotoxic payloads (56). The accelerated clearance of these short-lived mutants should produce a higher tumor-to-normal tissue ratio and fewer side effects.

Valency

Multivalent mAbs that hypercrosslink their target antigens can be more effective anti-tumor agents (9, 41). Initial studies to explore multivalency were carried out with tetravalent chemical homodimers, and these molecules displayed better *in vitro* and *in vivo* anti-tumor activity compared with their parental divalent monomers (41). However, because chemically generated homodimers are large, a tetravalent anti-human CD22 mAb with a single human Fc region and four murine Fv regions was generated (57, 58). It demonstrated improved anti-tumor activity in SCID mice xenografted with human B-lymphoma cells (unpublished data). This construct is not only smaller but also has a longer half life (57, 58).

Immunogenicity

The generation of an immune response against mAbs leads to rapid clearance and can induce side effects ranging from hypersensitivity to life-threatening anaphylactic reactions (59, 60). The transition from mouse to chimeric and later humanized mAbs was expected to dramatically decrease immunogenicity (61). Regardless, chimeric or humanized mAbs can still induce anti-Id responses. For example, a recombinant fully human IgG1 anti-TNF mAb (adalimumab) produced by phage display was immunogenic in some but not all patients (62, 63). Because variable regions of both mouse and human origin (idiotypes) are potentially immunogenic, this is not surprising (64). Moreover, it was reported that other factors could be involved in the immunogenicity of a mAb. These included physicochemical properties (e.g. stability and solubility), the dose and the route of injection, and concomitant treatments with other agents (65, 66).

Fc functions

Commonly used anti-tumor mAbs include intact IgG molecules as well as their antigen-binding fragments (67–71). Many attempts have been made to improve the anti-tumor activity of mAbs by decreasing their sizes and hence increasing tumor penetration. Antibody fragments generated by proteolysis or by genetic engineering have been tested both *in vitro* and *in vivo* (72–76). Among these mAbs fragments, Fab or Fab', single chain Fv (scFv) and disulfide bond-stabilized Fv (dsFv)

with a valency of one, or F(ab')₂ and diabodies with a valency of two have been used. These have lower MWs [ranging from 25 for scFv to 100 kDa for F(ab')₂] than the intact antibody (150 kDa). Hence, to increase their binding avidity, they have also been engineered into multivalent constructs (77, 78).

Unfortunately, even divalent antibody fragments have disadvantages. For example, the anti-tumor activity of most mAbs is dependent upon their effector functions and hence the presence of an Fc (49, 79, 80). The long half-life (T_{1/2}) of intact mAbs does generate a higher gradient concentration across cellular barriers with better penetration into the targeted tumor (81, 82). mAb fragments lacking an Fc cannot elicit ADCC and CDC and have very short half-lives. The uptake of the fragments into tumor is therefore markedly reduced as compared with intact antibodies (83, 84).

Antibody fragments with low MWs (scFv and dsFv) are currently used to target payloads to cells. These fragments are usually expressed as fusion proteins containing toxic fragments from *Pseudomonas* or diphtheria toxins. In contrast, minibodies, F(ab')₂, and CH₂-deleted antibody fragments have intermediate MWs and are conjugated to radionuclides (85–89). Because these immunoconjugates lack an Fc, they also have short half-lives and cannot bind to cells expressing FcγRs, resulting in fewer side effects.

In summary, intact 'naked' therapeutic antibodies with both effector functions and longer half-lives should outperform antibody fragments, while immunoconjugates should be effective even when an Fc is lacking. Because the presence of an Fc in a naked mAb is highly advantageous, efforts have been made to improve the anti-tumor activity of such mAbs by altering their Fc to confer improved effector functions and an even longer half life. To this end scFv and Fab fragments generated from phage display technology have been grafted onto an Fc (90–92).

Interaction of IgG with FcRs

The Fc region of IgG molecules is a symmetric homodimer of the carboxy-terminal portion of heavy chains and is composed of the CH₂ and CH₃ domains which are separated from the Fab by a flexible hinge region. The Fc region is stabilized by non-covalent interactions between domains. The Fc region interacts with FcRs to exert effector functions or to regulate the catabolism of IgG.

Interaction of IgG with FcγRs

FcγRs are broadly expressed as transmembrane glycoproteins on cells such as macrophages, NK cells, dendritic cells, B cells,

neutrophils, and mast cells. With the exception of FcγRII, FcγRs belong to the Ig superfamily and have extensive sequence homology. There are two classes of FcγRs, activating (FcγRI, FcγRIIA, and FcγRIII) and inhibitory (FcγRIIB) receptors (93). Interaction of IgGs with activating FcγRs triggers cell activation, while interaction with FcγRIIB inhibits cell activation. With the exception of B cells and NK cells, activating and inhibitory FcγRs are co-expressed on the same effector cells; thereby generating a threshold for cell activation. B cells express only the inhibitory FcγRIIB and therefore cannot be activated by endogenous IgG under physiological conditions. NK cells express the activating FcγRIII so that they can kill target cells independently of preactivation.

The FcγR binding of each IgG subclass varies, and hence, each exerts different effector functions. FcγRI (CD64) has a high affinity for IgG1 but not IgG2, while FcγRII (CD32) binds well to IgG3. FcγRI has a high affinity for both monomeric IgG ($K_a = 10^8 \text{ M}^{-1}$) and immune complexes. Because FcγRI is usually saturated by monomeric IgG, it cannot bind to immune complexes. Therefore, only high concentrations of immune complexes can activate FcγRI and elicit ADCC (94–96). In contrast, FcγRII and FcγRIII (CD16) have low affinities for monomeric IgG ($K_a = 10^6 \text{ M}^{-1}$) and are more critical for triggering effector functions, leading to anti-tumor activity (48, 97–100). Insights into the structure of different IgG subclasses and their interactions with FcγRs provide important insights into improving their effector functions. Usually, mAbs are genetically engineered to have increased affinities for the activating FcγRIII, and decreased affinities for the inhibitory FcγRIIB.

The amino acid residues in IgGs contributing to their direct interaction with FcγRs are located predominantly in the lower hinge region and are adjacent to the Cγ2 region. It has been demonstrated that residues 234–237 in the IgG molecule are required for binding to FcγRs (100–104) (Fig. 2). Mutations in these residues result in a considerable decrease in the affinity of IgG for FcγRII. In addition, the N-linked oligosaccharide at Asn297 is critical for the binding of IgG to all FcγRs (105, 106). Other important residues involved in IgG–FcγRs interactions are located in the Cγ2 domain (Asp265, Asp270, Ala327, Pro329, and Lys338) (107) (Fig. 2).

The importance of ADCC for anti-tumor mAbs

ADCC is dependent upon the ability of the FcγR-bearing cells of the innate immune system (e.g. NK cells, monocytes, macrophages, and granulocytes) to recognize the Fc domain of antibody bound to target cells (108). This recognition

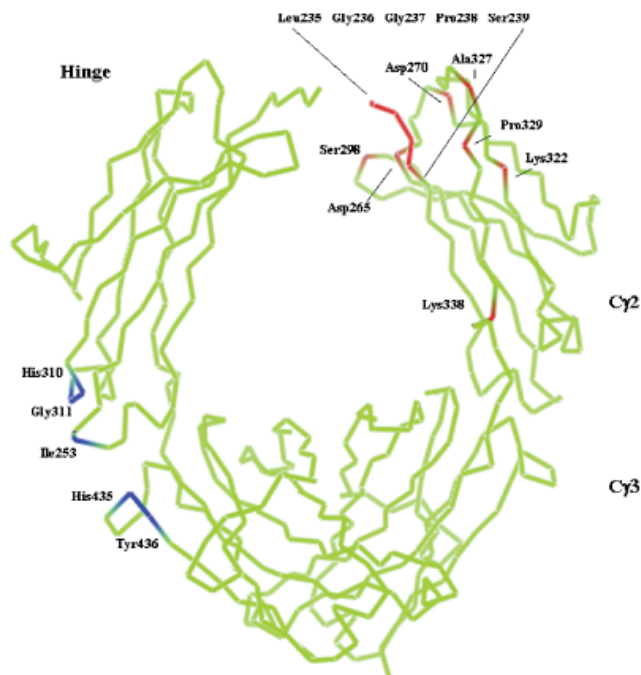


Fig. 2. Amino acid residues in the human IgG1 Fc involved in its interaction with FcγRs and/or C1q (red), and FcRn (blue). The binding sites (EU numbering) are represented on only one chain. The structure file was downloaded from PDB (Protein Data Bank), file # 1t89. The image was created using PyMOL™ 2006 DeLano Scientific LLC soft.

triggers effector cells to release cytoplasmic perforin, granzyme, and granzymes that induce apoptosis and lysis of target cells. The major effector cells in ADCC are NK cells which express both FcγRIIC (CD32c) and FcγRIIA (CD16a) (108–110). These FcγRs recognize the IgG1 and IgG3 subclasses and trigger cytotoxic effects *in vivo*. Studies demonstrating the loss of therapeutic activity of trastuzumab and rituximab in FcγR knockout mice have underscored the critical role of Fc–FcγR interactions in anti-tumor responses (48).

Engineering the Fc of a mAb to enhance ADCC

Amino acid mutations in the Fc
The identification of amino acid residues in an IgG1 that are critical for its binding to activating and inhibitory FcγRs has led to the generation and testing of many mutants that enhance or reduce the affinity of an IgG for these receptors (107). Mutations leading to increased binding to FcγRIII enhance ADCC *in vitro*. For example, triple amino acid substitution in alemtuzumab, rituximab, cetuximab, or trastuzumab (Ser298-Ala/Glu333Ala/Lys334Ala) increased their affinity for FcγRIIIA but not for FcγRIIB (107). Furthermore, the Fc variant Ser239Asp/Ile332Glu has improved ADCC (107). Fc variants of the trastuzumab exert enhanced FcγRIIIA-dependent ADCC *in vitro* (107, 111). Other mutants (Ser239Asp/

Ala330Leu/Ile332Glu and Ser239Asp/Ser298Ala/Ile332Ala) with better *in vitro* ADCC are under development (107).

By using surface display technology in yeast, amino acid residues in the Fc that are involved in enhanced binding of IgG to FcγRIIIA and reduced binding to FcγRIIB have been identified (112). Residues include Phe243, Asp270, Arg292, Ser298, Tyr300, Val305, Ala330, and Pro396. Fc variants containing individual amino acid mutations had minor increases in their binding to FcγRIIIA, but ADCC was not increased. Combining different mutations resulted in an Fc mutant (Phe243Leu/Arg292Pro/Tyr300Leu/Val305Ile/Pro396Leu) that showed a 100-fold increase in ADCC activity, and this was confirmed in three different mAbs. These mutants demonstrated an increased affinity for FcγRIIIA and a decreased affinity for FcγRIIB. Because the primary effector cells in ADCC are NK cells and these do not express FcγRIIB, optimal anti-tumor activity should be increased by designing mAbs that recruit not only NK cells but also effector cells expressing both activating and inhibitory FcRs, such as monocytes/macrophages. Despite encouraging results using these mutants, it is important to recognize that too many mutations have the potential to enhance the immunogenicity of mAbs, resulting in unpredictable biological consequences.

Modification of the carbohydrate moieties in the Fc
The second approach for enhancing the anti-tumor activity of mAbs involves modifying the carbohydrate moieties in the Fc. Some modifications appear to dramatically increase the affinity of the Fc for FcγRs. The IgG molecule contains two asparagine N-linked oligosaccharide sites in the Fc domain. The general structure of IgG N-linked oligosaccharides includes a core biantennary heptapolysaccharide containing N-acetylglucosamine (GlcNAc) and mannose attached to the Asn297 residue of Fc region (107, 113–115). Other polysaccharide variants include the presence or absence of bisecting GlcNAc fucose, galactose, and sialic acid. ADCC requires the presence of these oligosaccharides and is dependent upon changes in the structure of the oligosaccharide.

Previous studies have shown that removing the fucose moiety attached to the innermost GlcNAc of the biantennary complex-type oligosaccharides dramatically increases ADCC by improving the binding of the Fc to FcγRIIIA without impairing CDC activity (115–121). As compared with its parental mAb, a fucose-deficient IgG1 has been reported to have a 50-fold higher affinity for human FcγRIIIA (116). Furthermore, the *in vitro* ADCC activities of both a hypofucosylated humanized IgG1 anti-IL-5 and a chimeric IgG1 anti-CD20 were 50-fold higher than fully fucosylated IgG1 (116). The high affinity

binding between the FcγRIIIA and a non-fucosylated antibody is mediated by the interactions between the carbohydrate attached at Asn162 in the FcγR molecule and in regions of the Fc that are more accessible to the FcγR (122).

Recently, a study comparing three sialic acid variants of Rituximab demonstrated a reduction in ADCC that correlated with higher levels of sialylation (123). One explanation for this observation was that excess of sialic acids can reduce the flexibility of the IgG molecule and thus decrease its affinity for FcγRIIIA. However, reducing the flexibility of an IgG could potentially impair antigen binding; this remains to be evaluated.

Interestingly, other variations in the glycan structure of an Fc conferred by the removal of β 1,4)-linked galactose residues from Alemtuzumab and Rituximab have no effect on ADCC but decrease CDC activity by twofold (117).

Engineering host cells to express mAbs with enhanced effector functions has also been described. When Chinese hamster ovary (CHO) cells were transfected with the enzyme, β-(1,4)-N-acetylglucosaminyltransferase III (GnT-III) (124), the levels of bisected, non-fucosylated oligosaccharides per IgG molecule were increased, and the ADCC activity mediated by these mAbs was 100-fold higher. The content of fucose in IgG can be manipulated by α-1,6-fucosyltransferase (FUT8) knockout CHO cells lacking core-fucosyl transferase activity (119) or by constitutive expression of small interfering RNA against FUT8 (125). The expression of guanosine diphosphate (GDP)-mannose 4,6-dehydratase (GMD) is also related to fucosylation of IgG. Recently, it has been demonstrated that GMD-knockout CHO cells produce non-fucosylated IgG1 (126).

The hypothesis that ADCC should be enhanced by a combination of amino acid mutations in the Fc domain of IgG and by the ablation of fucosylation proved to be incorrect (127). Hence, when the previously described triple mutants of Rituximab (Ser239Asp/Ala330Leu/Ile332Glu and Ser239Asp/Ser298Ala/Ile332Ala) were expressed in non-fucosylated form in FUT8-knockout CHO cells, no enhancement of ADCC was observed. This observation led to the conclusion that the affinity of Fc for the FcγRIIIA does not anyways correlate with increases in ADCC.

Interaction of IgG with the complement system: the enhancement of complement activity by mutations in the Fc domain of IgG

The complement system consists of three pathways involving more than 30 soluble proteins. These proteins circulate in the plasma in their inactive forms and are subsequently

enzymatically activated (128). Complement can be activated by antigen–antibody complexes (classical pathway), by the absorption of C3 molecule onto the surface of pathogens (alternative pathway), and by mannose binding to bacterial surfaces containing mannans (129–132). These pathways lead to the formation of the membrane attack complex C5b-9, which forms pores in the plasma membranes of target cells and causes their lysis (133, 134). The initiation of the classical pathway is triggered by antigen–antibody complexes. The critical molecule for initiating the activation of this pathway is C1q, which binds to the Fc domain of IgG in immune complexes. After binding, a cascade of enzymatic events results in the formation of the membrane attack complex (135). Many mAbs approved by the FDA for cancer therapy can activate complement *in vitro* and mediate CDC. Among the human IgG subclasses, only IgG1 and IgG3 can initiate the complement cascade, and the amino acid residues responsible for C1q binding have been mapped by substituting amino acid residues with alanine (136).

To increase the ability of IgGs to recruit C1q, several IgG mutants have been developed. Four amino acid residues that are spatially close to each other in the Fc bind to C1q (Asp270, Lys322, Pro329, and Pro331) (Fig. 2). Two amino acid residues (Lys326 and Glu333) are located in the C1q binding epicenter of the CH₂ domain Rituximab (137). These residues do not interact with the oligosaccharide chains in the CH₂ domain. The substitution of Lys326 with nonpolar neutral amino acids (e.g. Ala, Met, or Trp) resulted in the enhancement of C1q binding and CDC activity. Different mutants have shown promising results: (i) Lys326Trp has threefold higher binding to C1q and twofold higher CDC activity; (ii) Glu333Ser has 1.7-fold higher CDC activity and a twofold increased affinity for C1q; (iii) Lys326Trp/Glu333Ser has a fivefold increase in the binding to C1q, suggesting that there is an additive effect of two sites that bind to C1q independently. However, CDC mediated by the Lys326Trp mutant and the double mutant were similar. This finding could be explained either by the presence of a threshold for CDC, or by the fact that the mutation(s) have different effects on subsequent steps in the complement cascade. Another important finding was that the double but not the single Lys326Trp mutant retained ADCC activity, suggesting that Lys326 is a critical amino acid involved in the interaction of IgG with FcγRs.

The role of the hinge region of human IgG1 in its ability to mediate effector function has also been studied (138). Twenty-six upper and middle hinge variants of a humanized IgG1 anti-EphA2 (tyrosine kinase receptor) have been generated by site-directed mutagenesis. IgG containing mutations in the upper hinge region had increased C1q binding and enhanced

CDC (e.g. Lys222Trp/Thr223Trp, Cys220Asp/Asp221Cys, Cys220Asp/Asp221Cys/Lys222Trp/Thr223Trp, Lys222Trp/Thr223Trp/His224Trp, and Asp221Trp/Lys222Trp), while IgG containing mutations in the middle hinge had impaired C1q binding, confirming previous studies that suggested that opening the bridge formed by N-terminal cysteine residue in the upper hinge prevents complement binding (139, 140). The mutants did not show any significant difference in binding to FcγRIIIAs. When mutations were performed in the middle hinge, a dramatic decrease in FcγRIIIA binding was observed. This finding led to the conclusion that the content, length, and flexibility of the middle hinge are critical for the affinity of the Fc for the FcγRIIIA and hence for ADCC. Taken together, studies performed on both the CH₂ domain and the hinge region led to the hypothesis that combining these two approaches should result in increased affinity of IgG1 for C1q. Hence, the design of mAbs with improved CDC activity is an important strategy for improving the cytotoxicity of a mAb, although there are no published reports of *in vivo* studies using this strategy.

It is known that there are many mechanisms that downregulate complement activation, such as the expression of membrane complement regulatory protein (mCRPs) (e.g. CD46, CD55, and CD59). These proteins can protect both normal and tumor cells from lysis mediated by the complement system. Bispecific mAbs that are antagonists for mCRPs and tumor antigen are therefore being developed (141–143).

Interaction of IgG with FcRns

The FcRn is a heterodimer of a major histocompatibility complex class I-like heavy chain and β2-microglobulin (β2 m) that is expressed on many types of cells including vascular endothelial cells, antigen-presenting cells, and epithelial cells (144–148). FcRns transport IgG across the placenta and also play crucial roles in maintaining the concentration of IgG in serum. FcRns bind to IgG with nanomolar affinity under acidic conditions (pH ≤ 6.5) but not at a physiological pH (149–152). Following nonspecific pinocytosis into FcRn-expressing cells, IgG is transported into acidified endosomes (pH 6.0), where IgG can bind to the FcRn. The IgG–FcRn complexes are then salvaged and transcytosed to the cell surface, where IgG molecules are released into the extracellular space due to the neutral pH of serum (pH 7.4). IgG molecules that do not bind to FcRns enter the lysosomal pathway and are degraded (Fig. 3).

FcRns bind to a region of the IgG Fc that is distinct from their FcγRs binding sites (107, 153). It has been demonstrated

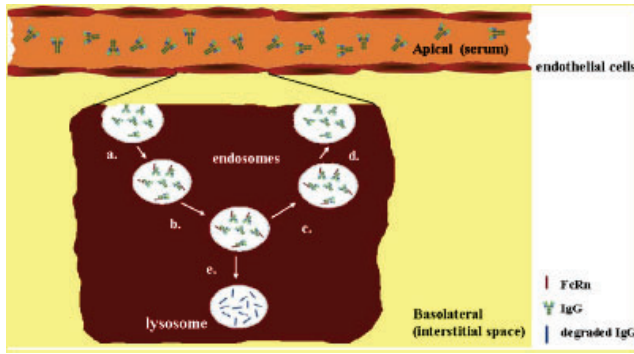


Fig. 3. Proposed mechanism for the catabolism of IgG in FcRn-expressing endothelial cells. (A) Uptake, (B) binding, (C) recycling, (D) release, (E) degradation of the unbound IgG [modified after Ghetie V and Ward ES, *Ann Rev Immunol* 2000 (217)].

Table 3. Amino acid residues involving in human IgG–FcRn interaction

Human IgG	Human FcRn
Ile253	Trp133
His310	Glu117
Gln311	Glu118
His435*	Asp132
Tyr436†	Leu137

*In IgG1, IgG2 and IgG4 allotypes; the residue in IgG3 is Arg.

†In IgG1, IgG2 and IgG4 allotypes; the residue in IgG3 is Phe.IgG, immunoglobulin.

that key amino acid residues involved in IgG–FcRn interactions are located at the interface between the $\text{C}\gamma 2$ and $\text{C}\gamma 3$ domains (Fig. 2). Amino acid residues Ile253 and His310 are highly conserved across all human IgG subclasses and play crucial roles in IgG–FcRn interactions (Table 3). In addition, His435 is also involved in regulating IgG–FcRn interactions. Hydrophobic interactions between the Ile253 of IgG and the Trp133 of the FcRn play essential roles in IgG–FcRn interactions. The presence of the weak basic histidine residues in the Fc and acidic residues in the FcRn suggests that electrostatic forces may mediate such interactions. In addition, other residues surrounding these critical positions can either directly affect the interactions between Fcs and FcRns or indirectly regulate this interaction by stabilizing the conformation of the $\text{C}\gamma 2$ – $\text{C}\gamma 3$ interface. In IgG1, these residues include Pro247, Lys248, Leu251, Thr250, Met252, Ser254, Arg255, Thr256, Pro257, Lys288, Thr307, Glu380, Met428, Ser415, His433, and Asn434 (154).

Because FcRns act as salvage receptors that regulate IgG catabolism, interactions between IgGs and FcRn are of major importance in controlling the concentrations of serum IgG. Indeed, most approaches aimed at improving the

pharmacokinetics of anti-tumor mAbs have focused on regulating the affinity of mAbs for FcRns.

Approaches for decreasing the *in vivo* half-lives of IgG immunoconjugates

IgG immunoconjugates have been designed to decrease their *in vivo* half-lives. Decreasing the half-life of IgG immunoconjugates should be advantageous because the excess of unbound ligand is quickly eliminated from the circulation and thus diminishes their side effects. Although the short half-life of murine IgG ensures the rapid elimination of immunoconjugates, the immunoconjugates containing murine mAbs are immunogenic to humans and cannot be used repeatedly (33, 34, 155). Thus, short-lived IgG immunoconjugates containing chimeric or humanized antibodies would be preferable and, therefore, different approaches have been used to reduce their half-lives.

Truncation of the Fc

Truncating all or part of the Fc portion of IgG will diminish IgG–FcRn interactions and decrease its half-life (156–158). Such truncated molecules include Fab, F(ab')_2 and scFv, as well as minibody and CH_2 -deleted antibody fragments. Because of their small molecular size, scFv (MW = 25 kDa) and Fab (MW = 50 kDa) are cleared rapidly by the kidney. Minibodies, F(ab')_2 and CH_2 -deleted mAbs have intermediate MWs (> 70 kDa), thereby avoiding rapid clearance and conferring with longer retention times and better tumor uptake as compared with scFvs and Fabs. In general, the half-lives of these Fc-truncated antibody fragments are shorter than 30 h (84, 159–162).

FcRn blockers

The use of FcRn blockers in conjunction with mAb therapy can prevent the binding of IgG immunoconjugates to FcRns, thereby increasing their clearance. For example, it has been demonstrated that serum concentrations of endogenous IgGs can be decreased by injecting human IgG mutants that have higher affinities for FcRn at both pH 6.0 and 7.2 (163). Hence, for cancer imaging and therapy, transient blockade of FcRns should accelerate the clearance of IgG immunoconjugates from normal tissues after tumor targeting and prevents normal-tissue toxicity while preserving the efficacy of anti-tumor immunoconjugates. In addition, this approach ensures that the FcRn can recover to maintain physiological concentrations of IgG in serum. Therefore, blocking the FcRn by co-injecting immunoconjugates with IgG is a viable option (56, 164). The

advantage of this approach is that each co-injected mAb itself does not need to be altered.

Mutations in the Fc

The half life of IgG immunoconjugates can also be decreased by altering the amino acid residues involved in the interaction of IgGs with the FcRns. Mutations generally involve residues located in the CH₂–CH₃ cleft (56, 165, and our unpublished data).

Five mutants (Ile253Ala, His310Ala, His435Gln, His435Arg, and His310Ala/His435Gln) of a chimeric mAb directed against carcinoembryonic antigen have been produced using a scFv-Fc, and these mutants have been compared with the wildtype molecule for their activities and pharmacokinetics (165). In BALB/c mice, these constructs had a decrease in blood clearance rates. The wildtype scFv-Fc showed the longest half-life ($T_{1/2\beta}$ = 289 h), while the His310Ala/His435Gln double mutant exhibited the shortest half-life ($T_{1/2\beta}$ = 7.96 h). Tumor targeting by the wildtype and mutant molecules has also been evaluated in athymic mice xenografted with the tumor cells expressing carcinoembryonic antigen. The wildtype scFv-Fc and slow clearing mutant His435Gln ($T_{1/2\beta}$ = 52.6 h) required longer times to localize in the tumor and to be cleared from circulation. The Ile253Ala and His310Ala mutants had intermediate clearance rates, while the shortest-lived His310Ala/His435Gln double mutant quickly localized in tumor sites and was rapidly cleared from the blood and tissues. Therefore, the double mutant produced clearer images. In addition, the wildtype antibody exhibited the highest tumor uptake at 48 h, while the His310Ala/His435Gln had the best tumor/normal tissue ratio. Thus, it is possible to tailor the Fc–FcRn interaction to achieve improved tumor/normal tissue targeting.

The anti-tumor activity of an immunotoxin (IT) with a decreased half-life was investigated by the His435Ala mutation of a chimeric anti-CD22 antibody conjugated to recombinant ricin toxin A chain. The IT mutant has a reduced affinity for human and rat FcRns and a decreased half-life in mice ($T_{1/2\beta}$ = 62.1 ± 1.12 versus 117 ± 10.0 h for the IT wildtype). In addition, the IT mutant and the wildtype control exhibited comparable *in vitro* anti-tumor activities, suggesting that fully active chimeric mAbs and IgG immunoconjugates containing an intact Fc with alterations in serum half-life can be constructed and should be further evaluated (56).

Mutagenesis of the Fc to increase the half-life of naked mAbs

Because the IgG–FcRn interaction is pH dependent, His310 and His435 in the Fc should be preserved when mutagenesis is

performed to increase the half life of a mAb. In human IgG, amino acid residues located at the interface or near the FcRn-binding sites are candidates for mutation, because these residues can directly alter the interaction of IgG with the FcRn. In addition, residues buried between the C γ 2 and C γ 3 domains are potential choices for mutagenesis, because these residues have an impact on the flexibility of the C γ 2–C γ 3 interface. Hence, mutagenesis of these residues may ‘optimize’ the interaction of Fcs with FcRns (154).

The residues located at the interface of Fc–FcRn have been chosen to produce mutants with longer half-lives. Groups of mutants of a murine IgG1 Fc fragment were obtained using phage display methodology. The Thr252Leu/Thr254Ser/Thr256Phe triple mutant had increased affinity for murine FcRn (K_D = 2.16 nM for the mutant versus 7.44 nM for the wildtype). In mice, this mutant exhibited a half-life of 153 versus 93 h for the wildtype IgG1 Fc (166).

Extensive analysis of the interactions between human or mouse FcRns and IgGs from various species has been carried out using surface plasmon resonance (167). Mouse FcRns bind to all IgGs analyzed, while human FcRn fails to bind or binds weakly to mouse IgG. These results provide the molecular explanation of the short half-life of mouse mAbs in humans and indicate that the pharmacokinetics of therapeutic mAbs should be tested using an appropriate animal model. Therefore, human FcRn transgenic mice or cynomolgus monkeys are more suitable models for the analysis of the pharmacokinetics of chimeric and humanized antibodies and their mutants. For example, a triple mutation (Met252Tyr/Ser254Thr/Thr256Glu) of a humanized anti-respiratory syncytial virus mAb (MEDI-524) was screened from the phage library of the human IgG1 Fc. The mutant exhibited a 10-fold increase in binding to the FcRns of both cynomolgus monkeys and humans at pH 6.0, but it did not exhibit a prolonged half-life in BALB/c mice, even though its half-life was increased by nearly fourfold in cynomolgus monkeys (168, 169).

Various mutants of human IgG designed to increase their affinity for the human FcRn have shown a 10-fold improvement in binding (170). Molecular models of the human IgG2 Fc–FcRn complex were generated based on the crystal structures of the rat Fc–FcRn complex. These models guided the selection of residues 250, 314, and 428 of the human IgG2 heavy chain for site-directed mutagenesis. Because the amino acids at these positions are located near the Fc–FcRn interface but do not make direct contact with FcRn, they do not directly contribute to the pH-dependent interaction between the Fc and the FcRn. However, random amino acid substitutions at these positions may change the

conformation of amino acids in the Fc that do interact with FcRn and thus, alter the affinity of IgG for the FcRn. Based on this hypothesis, random site-directed mutagenesis was performed and in competitive binding assays mAbs with mutations at positions 250 and 428 were stronger competitors than the wildtype mAb, indicating that these mutants bind more avidly to FcRns at pH 6.0. The best mutants were Thr250Gln, Met428Leu or their combination. At pH 6.0, their affinity for the human FcRn was increased by 3-, 7-, and 28-fold, respectively, while at neutral pH this binding was lost. As compared with the wildtype mAb, the half-lives of the Met428Leu and the Thr250Gln/Met428Leu mutants in rhesus monkeys were increased by approximately 1.8- and 1.9-fold, respectively. However, in the same study, none of the mutations at position 314 resulted in increased binding of IgGs to FcRns.

Using a similar molecular model, IgG subclasses of an anti-human hepatitis B virus mAb were investigated (171). Thr250Gln and Met428Leu mutations in IgG1, IgG2, and IgG4 antibodies showed 30-fold increased binding affinities for both human and rhesus FcRns as compared to their wildtype counterparts, while the $T_{1/2}\beta$ of the Thr250Gln/Met428Leu double mutant of IgG1 in rhesus monkeys was increased by 2.5-fold. An enhanced affinity of IgG1, IgG2, and IgG4 variants of an anti-HLA-DR- β chain mAb for human and rhesus FcRns was also demonstrated. Interestingly, site-directed mutagenesis did not impair antigen binding, ADCC, or CDC, indicating that the Thr250Gln and Met428Leu mutations had no effect on the interactions of mAbs with Fc γ Rs or complement.

The translation of an increased binding affinity for FcRn to improved pharmacokinetics is controversial. The Thr250Gln/Met428Leu mutant of a humanized anti-TNF- α mAb had a 40-fold increase in affinity for the FcRn of cynomolgus monkeys at pH 6.0 as compared with its wildtype counterpart and it maintained the pH binding dependency. However, the mutant failed to show a longer half-life in cynomolgus monkeys (172). In addition, Pro257Ile/Asn434His, Asp376Val/

Asn434His, and Pro257Ile/Gln311Ile variants of the same mAb exhibited ~15- to 197-fold increases in binding to human, cynomolgus monkey, or mouse FcRns at pH 6.0 but did not bind at pH 7.4. These mutants did not show increased half-lives when they were tested in cynomolgus monkeys or mice.

Single mutations in Thr370Ala, Glu380Ala, or Asn434Ala in human IgGs showed increases in affinity for human FcRn of 1.8-, 2.2- and 3.5-fold, respectively, while the triple mutant resulted in an 11-fold increase (107). However, no increase in the half-life of these mutants in mice expressing murine FcRn and only a marginal increase in human FcRn transgenic mice was observed for the Asn434Ala and the triple mutant (173). Therefore, in addition to binding to FcRn, other factors such as resistance to proteolysis and the binding of mAbs to the target antigens should be considered when an effort is made to prolong the half-lives of mAbs.

Concluding remarks

Based on new insights gained by the study of the cellular and molecular mechanisms that are involved in the interaction of IgG with FcRs and complement, glycoengineering and protein engineering have been used to create a new generation of therapeutic mAbs. Such mAbs are characterized by enhanced effector functions or optimized pharmacokinetics, and several have shown promising results in preclinical studies. However, the *in vivo* anti-tumor activity of most of these new mAbs has yet to be investigated. In addition, due to the difference in the interactions of human IgG with the FcRns from different species, improved pharmacokinetic characteristics must be confirmed in humans. Since the amino acid residues involved in the interaction of IgG molecule with murine and human Fc γ Rs and FcRn differ, mAbs containing mutations at both binding sites might demonstrate enhanced effector functions as well as optimized pharmacokinetics.

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