1
Introduction: Antibody Structure and Function

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1.1
Introduction to Antibodies

Antibodies, a central part of humoral immunity, have increasingly become a dominant class of biotherapeutics in clinical development and are approved for use in patients. As with any successful endeavor, the history of monoclonal antibody therapeutics benefited from the pioneering work of many, such as Paul Ehrlich who in the late nineteenth century demonstrated that serum components had the ability to protect the host by "passive vaccination" [1], the seminal invention of monoclonal antibody generation using hybridoma technology by Kohler and Milstein [2], and the advent of recombinant technologies that sought to reduce the murine content in therapeutic antibodies [3].

During the process of generation of humoral immunity, the B-cell receptor (BCR) is formed by recombination between variable (V), diversity (D), and joining (J) exons, which define the antigen recognition element. This is combined with an immunoglobulin (Ig) constant domain element (μ for IgM, δ for IgD, γ for IgG (gamma immunoglobulin), α for IgA, and ε for IgE) that defines the isotype of the molecule. Sequences for these V, D, J, and constant domain genes for disparate organisms can be found through the International ImMunoGeneTics Information System® [4]. The different Ig subtypes are presented at different points during B-cell maturation. For instance, all naïve B cells express IgM and IgD, with IgM being the first secreted molecule. As the B cells mature and undergo class switching, a majority of them secrete either IgG or IgA, which are the most abundant class of Ig in plasma.

Characteristics like high neutralizing and recruitment of effector mechanisms, high affinity, and long resident half-life in plasma make the IgG isotype an ideal candidate for generation of therapeutic antibodies. Within the IgG isotype, there are four subtypes (IgG1–IgG4) with differing properties (Table 1.1). Most of the currently marketed IgGs are of the subtype IgG1 (Table 1.2).

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Table 1.1 Subtype properties.

Property	IgG1	IgG2	IgG3	IgG4
Heavy chain constant gene	γ1	γ2	γ3	γ4
Approximate molecular weight (kDa)	150	150	170	150
Mean serum level (mg/ml)	9	3	1	0.5
Half-life in serum (days)	21	21	7	21
ADCC	+	−	+	+/−
CDC	++	+	+++	−
Number of disulfides in hinge	2	4	11	2
Number of amino acids in hinge	15	12	62	12
Gm allotypes	4	1	13	−
Protein A binding	+++	+++	+	+++
Protein G binding	+++	+++	+++	+++

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

Table 1.2 Marketed antibodies and antibody derivatives by target.

Trade name	International non-proprietary name	Target	Type	Indication
Benlysta®	Belimumab	BLyS	Human IgG1λ	SLE
Soliris®	Eculizumab	C5	Humanized IgG2/4	PNH
Raptiva®	Efalizumab	CD11a	Humanized IgG1κ	Psoriasis
Amevive®	Alefacept	CD2	CD2-binding domain of LFA3–IgG1 Fc fusion	Psoriasis
Rituxan®	Rituximab	CD20	Chimeric IgG1κ	NHL, CLL, RA, GPA/MPA
Zevalin®	Ibritumomab tiuxetan	CD20	Murine IgG1κ–Y90/In111 conjugate	NHL
Bexxar®	Tositumomab-I131	CD20	Murine IgG2aλ–I131 conjugate	NHL
Arzerra®	Ofatumumab	CD20	Human IgG1κ	CLL
Orthoclone-OKT3®	Muromonab-CD3	CD3	Murine IgG2a	Transplant rejection
Adcetris®	Brentuximab vedotin	CD30	Chimeric IgG1κ-conjugated MMAE	Hodgkin's lymphoma
Mylotarg®	Gemtuzumab ozogamicin	CD33	Humanized IgG4κ–calicheamicin conjugate	Leukemia
Campath-1H®	Alemtuzumab	CD52	Humanized IgG1κ	Leukemia
Orencia®	Abatacept	CD80/CD86	CTLA4–IgG1 Fc fusion	RA
Nulojix®	Belatacept	CD80/CD86	CTLA4–IgG1 Fc fusion	Transplant rejection
Yervoy®	Ipilimumab	CTLA4	Human IgG1κ	Metastatic melanoma

Erbitux®	Cetuximab	EGFR	Chimeric IgG1κ	Colorectal cancer
Vectibix®	Panitumumab	EGFR	Human IgG2κ	Colorectal cancer
Removab®	Catumaxomab	EpCAM/CD3	Rat IgG2b/mouse IgG2a	Malignant ascites
ReoPro®	Abciximab	gPIIb/IIIa	Chimeric Fab	PCI complications
Herceptin®	Trastuzumab	Her2	Humanized IgG1κ	Breast cancer
Kadcyla®	Trastuzumab emtansine	Her2	Humanized IgG1κ–DM1 conjugate	Breast cancer
Perjeta®	Pertuzumab	Her2	Humanized IgG1κ	Breast cancer
Xolair®	Omalizumab	IgE	Humanized IgG1κ	Asthma
Ilaris®	Canakinumab	IL-1b	Human IgG1κ	CAPS, FCAS, MWS
Arcalyst®	Rilonacept	IL1	IL1R1–IL1RAcP–IgG1 Fc fusion	CAPS
Stelara®	Ustekinumab	IL12/IL23	Human IgG1κ	Psoriasis
Zenapax®	Daclizumab	IL2ra	Humanized IgG1	Transplant rejection
Simulect®	Basiliximab	IL2ra	Chimeric IgG1κ	Transplant rejection
Actemra®	Tocilizumab	IL6r	Humanized IgG1κ	RA
Tysabri®	Natalizumab	LFA4	Humanized IgG4κ	MS
Prolia®	Denosumab	RANKL	Human IgG2κ	Bone metastases
Synagis®	Pavilizumab	RSV F protein	Chimeric IgG1κ	RSV
Remicade®	Infliximab	TNFα	Chimeric IgGκ	RA
Enbrel®	Etanercept	TNFα	TNFrII–p75 ECD–IgG1 Fc fusion	RA
Humira®	Adalimumab	TNFα	Human IgG1κ	RA, Crohn's disease
Cimzia®	Certolizumab pegol	TNFα	Humanized IgG1κ Fab–PEG conjugate	RA
Simponi®	Golimumab	TNFα	Human IgG1κ	RA, PA, AS
Nplate®	Romiplostim	TPOr	Peptide–IgG1 Fc fusion	TCP, UC
Avastin®	Bevacizumab	VEGF	Humanized IgG1κ	Colorectal cancer
Lucentis®	Ranibizumab	VEGF	Humanized IgG1κ Fab	wAMD
Eylea®	Aflibercept	VEGF-A	VEGFr1 and VEGFr2–IgG1 Fc fusion	wAMD

Abbreviations: AS, ankylosing spondylitis; CAPS, cryopyrin-associated periodic syndrome; CLL, chronic lymphocytic leukemia; FCAS, familial cold autoinflammatory syndrome; GPA/MPA, granulomatosis with polyangiitis (Wegener's granulomatosis)/microscopic polyangiitis; MS, multiple sclerosis; MWS, Muckle–Wells syndrome; NHL, non-Hodgkin's lymphoma; PA, psoriatic arthritis; PCI, percutaneous coronary intervention; PNH, paroxysmal nocturnal hemoglobinuria; RA, rheumatoid arthritis; RSV, respiratory syncytial virus; SLE, systemic lupus erythematosus; TCP, thrombocytopenia; UC, ulcerative colitis; wAMD, neovascular (wet) age-related macular degeneration.

The ability of antibodies to recognize their antigens with exquisite specificity and high affinity makes them an attractive class of molecules to bind extracellular targets and generate a desired pharmacological effect. Antibodies also benefit from their ability to harness an active salvage pathway, mediated by the neonatal Fc receptor (FcRn), thereby enhancing their pharmacokinetic (PK) life span and mitigating the need for frequent dosing. The antibodies and antibody derivatives approved in the United States and the European Union (Table 1.2) span a wide range of therapeutic areas, including oncology, autoimmunity, ophthalmology, and transplant rejection. They also harness disparate modes of action like blockade of ligand binding and subsequent signaling, and receptor and signal activation, which target effector functions (antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)), and delivery of cytotoxic payload.

Antibodies are generated by the assembly of two heavy chains and two light chains to produce two antigen-binding sites and a single constant domain region (Figure 1.1, panel a). The constant domain sequence in the heavy chain designates the subtype (Table 1.1). The light chains can belong to two families (λ and κ), with most of the currently marketed antibodies belonging to the κ family.

The antigen-binding regions can be derived by proteolytic cleavage of the antibody to generate antigen-binding fragments (Fab) and the constant fragment (Fc, also known as the fragment of crystallization). The Fab comprises the variable regions (variable heavy (VH) [11] and variable light (VL)) and constant regions (C_H1 and Cκ/Cλ). Within these variable regions reside loops called complementarity determining regions (CDRs) responsible for direct interaction with the antigen (Figure 1.1, panel b). Because of the significant variability in the number of amino acids in these CDRs, there are multiple numbering schemes for the variable domains [12,13] but only one widely used numbering scheme for the constant domain (including portions of the C_H1, hinge, and the Fc) called the EU numbering system [14].

There are two general methods to generate antibodies in the laboratory. The first utilizes the traditional methodology employing immunization followed by recovery of functional clones either by hybridoma technology or, more recently, by recombinant cloning of variable domains from previously isolated B cells displaying and expressing the desired antigen-binding characteristics. There are several variations of these approaches. The first approach includes the immunization of transgenic animals expressing subsets of the human Ig repertoire (see review by Lonberg [15]) and isolation of rare B-cell clones from humans exposed to specific antigens of interest [16]. The second approach requires selecting from a large *in vitro* displayed repertoire either amplified from natural sources (i.e., human peripheral blood lymphocytes in Ref. [17]) or designed synthetically to reflect natural and/or desired properties in the binding sites of antibodies [18,19]. This approach requires the use of a genotype–phenotype linkage strategy, such as phage or yeast display, which allows for the recovery of genes for antibodies displaying appropriate binding characteristics for the antigen.

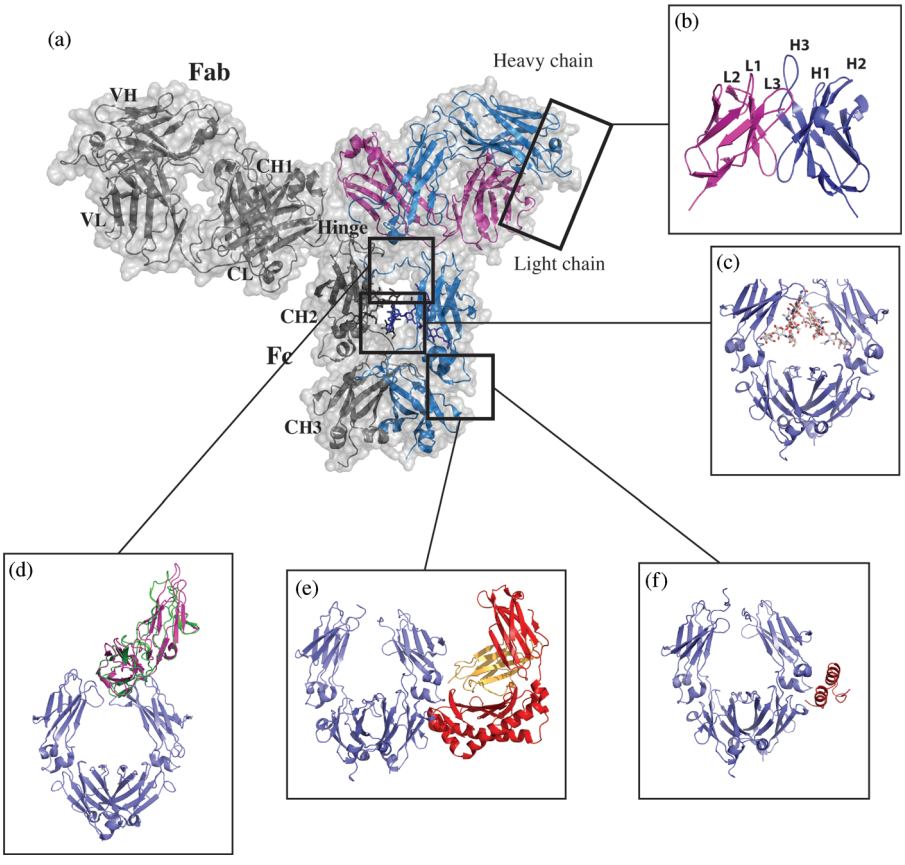

Figure 1.1 Structure and features of the IgG and its interactions. (a) The structure of a full-length IgG is shown in ribbon representation with transparent molecular surface. One heavy chain is shown in blue and one light chain in magenta. The other heavy chain and light chain are shown in gray for clarity. In this orientation, two Fab domains sit on top of the Fc domain and are connected in the middle by the hinge region. The Fab domain is composed of the heavy chain V_H and C_H1 domains and the light chain V_L and C_L domains—Protein Data Bank (PDB) [5] code 1HZH [6]. (b) Each variable domain contains three variable loops (L1–L3 on light chain and H1–H3 on heavy chain) that make up the antigen-binding site—PDB code 1HZH [6]. (c) The Fc region is composed of the dimer of C_H2 and C_H3 domains. The C_H3 domains form a tight interaction while the C_H2 domains interact through protein–protein, protein–carbohydrate, and carbohydrate–carbohydrate contacts—PDB code 1HZH [6]. (d) The hinge region is composed of a flexible region covalently tied together through disulfide bridges. Structures of the FcγRIIIa and FcγRIIa bound to the Fc are shown. The structures reveal that both receptors bind to the C_H2 domain near the hinge and carbohydrates and upon their binding create an asymmetry such that the second FcγR is unable to bind. In this panel, FcγRIII is shown in green, and the FcγRII is shown in purple—PDB codes 3RY6 [7] and 1T83 [8]. (e) The crystal structure of the complex between the Fc and FcRn reveals that FcRn binds between the C_H2 and C_H3 domains in the Fc. FcRn chains are shown in red and orange—PDB code 1FRT [9]. (f) Interestingly, the same region also binds to bacterial Protein A commonly used for purification—PDB code 1FC2 [10].

1.2 General Domain and Structure of IgG

Topologically, the IgG is composed of two heavy chains (50 kDa each) and two light chains (25 kDa each) with total molecular weight of approximately 150 kDa. Each heavy chain is composed of four domains: the variable domain (VH), C_H1, C_H2, and C_H3. The light chain is composed of variable domain (VL) and constant domain (CL). All domains in the IgG are members of the Ig-like domain family and share a common Greek-key beta-sandwich structure with conserved intradomain disulfide bonds. The CLs contain seven strands with three in one sheet, and four in the other, while the VLs contain two more strands, resulting in two sheets of four and five strands.

The light chain pairs up with the heavy chain VH and C_H1 domains to form the Fab fragment, while the heavy chain C_H2 and C_H3 domains dimerize with additional heavy chain C_H2 and C_H3 domains to form the Fc region (Figure 1.1, panel c). The Fc domain is connected to the Fab domain via a flexible hinge region that contains several disulfide bridges that covalently link the two heavy chains together. The light chain and heavy chains are also connected by one disulfide bridge, but the connectivity differs among the IgG subclasses (Figure 1.2). The overall structure of IgG resembles a Y-shape, with the Fc region forming the base while the two Fab domains are available for binding to the antigen [6]. Studies have shown that in solution the Fab domains can adopt a variety of conformations with regard to the Fc region.

1.2.1 Structural Aspects Important for Fc Fusion(s)

1.2.1.1 Fc Protein–Protein Interactions

While the Fab region of an antibody is responsible for binding and specificity to a given target, the Fc region has many important functions outside its role as a structural scaffold. The Fc region is responsible for the long half-life of antibodies as well as for their effector functions including ADCC, CDC, and phagocytosis [20].

The long half-life of human IgGs relative to other serum proteins is a consequence of the pH-dependent interaction with the FcRn [21–23]. In the endosome, FcRn binds to the Fc region and recycles the antibody back to the plasma membrane, where the increase in pH releases the antibody back to the serum, thus rescuing it from degradation. The details of FcRn binding and its effects on antibody pharmacokinetics, including results from modulating FcRn interaction by protein engineering, are discussed in Section 1.3.3. One FcRn binds between the C_H2 and C_H3 domains of an Fc dimer half (Figure 1.1, panel e) [21]; therefore, up to two FcRns can bind to a single Fc.

Fc region is also responsible for binding to bacterial Protein A [10] and Protein G [24], which are commonly used for purification of Fc-containing proteins. Although Protein A binds to Fc mainly through hydrophobic interactions and

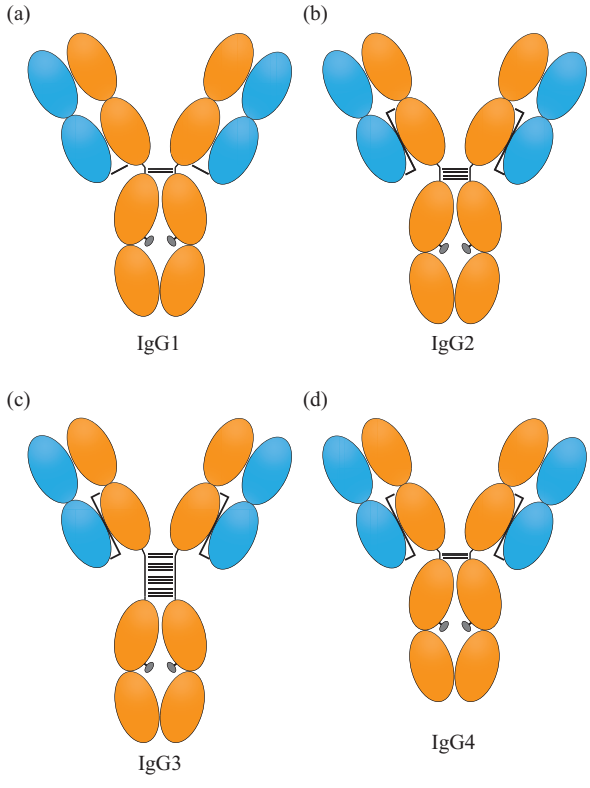

Figure 1.2 Interchain disulfide topology in human IgG subclasses. Only H–H hinge and H–L chain disulfides are shown. (a) IgG1, (b), IgG2, (c) IgG3, and (d) IgG4.

Protein G through charged and polar interactions, Proteins A and G bind to a similar site on Fc domain and compete with each other (Figure 1.1, panel f). Interestingly, the binding occurs between the C_H2 and C_H3 domains of the Fc and largely overlaps with the FcRn binding site.

ADCC function is mediated by the interaction of the Fc region with Fcγ receptors (FcγRs). Biochemical data and structures of Fc in complex with FcγRIII and FcγRII reveal that the FcγRs bind to the combination of the Fc C_H2 domain and the lower hinge region (Figure 1.1, panel d) [7,8,25]. Members of the Fcγ family have been found to bind to the same region of Fc [20,26,27] and form a 1 : 1 asymmetric complex where one FcγR interacts with the dimer of Fc. The binding of one FcγRIII to Fc induces asymmetry in the Fc region and prevents a second interaction. While the detailed structural understanding is not available for the Fc–C1q interaction, biochemical data suggest that C1q binds mainly to the C_H2 domain with an overlapping, but nonidentical, binding site of FcγRIII [28]. The

details of the interaction between the Fc and Fcγ receptors, as well as the engineering of effector function, are further discussed in Section 1.4.2.1.

1.2.1.2 Fc Glycosylation

The Fc region of IgG has a conserved glycosylation site in the C_H2 domain at position N297 (Figure 1.1, panel c). Glycosylation of the C_H2 domain is important in achieving optimal effector function [29] and complement activation; it also contributes to overall IgG stability [30]. Antibodies purified from human serum have been found to contain heterogeneous oligosaccharides where each C_H2 domain can contain one of many potential glycans [31]. Therapeutic Fc-containing proteins that are expressed in Chinese hamster ovary (CHO) or human embryo kidney 293 (HEK293) cells typically contain a mixture of glycoforms, with G0F being the most abundant, followed by G1F and G2F [32,33]. The attachment of the glycans at position Asn297 in the C_H2 domain positions the carbohydrates to interact with each other and to form a part of the Fc dimer interface. Because of carbohydrate sequestration into the space between the two C_H2 domains and significant carbohydrate–carbohydrate and carbohydrate–protein contacts, the carbohydrates in the Fc crystal structures are relatively well ordered.

The glycosylation of the Fc has been found to influence biological activity as well as stability of IgGs [34,35]. The removal of the core fucose enhances ADCC activation of FcγRIIIa on natural killer (NK) cells but does not change the binding of FcγRI or C1q [36]. Increased ADCC has also been observed with the presence of bisecting *N*-acetylglucosamine in the context of fucosylated IgG, although the effect appears to be smaller than removal of the core fucose [37]. Sialylated IgGs have been suggested to enhance anti-inflammatory properties [38]; however, more work is needed to understand this effect and potential mechanism.

1.2.1.3 Hinge and Interchain Disulfide Bonds

The hinge region of human IgGs (IgG1, IgG2, and IgG4) differs between the subtypes both in the hinge length (12–15 residues) and in number of disulfides linking the two heavy chains together (2–4 residues) (Figure 1.2). In addition, the position of the light chain–heavy chain linkage differs among the human IgG subtypes (Figure 1.2). In human IgG1, two disulfides link the heavy chains together while human IgG2 contains four disulfides and a shorter hinge. The presence of an increased number of disulfides as well as a shorter hinge likely decreases the flexibility of hIgG2 Fab regions relative to hIgG1. The hinge can have a profound impact on antibody properties. For example, the sequence in the hinge near the disulfides has been found to be important in the ability of IgG4s to exchange half molecules *in vivo* and under certain conditions *in vitro* [39,40]. The absence of one of the proline residues in the hinge of IgG4 coupled with substitution in the C_H3 domain allows IgG4 to form half-antibodies and form bispecific antibodies by exchanging with other IgG4s (Figure 1.2).

1.3 The Neonatal Fc Receptor

1.3.1 FcRn Function and Expression

One major characteristic of IgG, which differs from other Ig isotypes and most of the other serum proteins, is its long serum half-life. Typically, serum proteins and other Ig isotypes have half-lives of <1 week, for example, fibrinogen (1–3 days), IgD (2–5 days), IgM (4–6 days), IgA (3–7 days), and haptoglobin (~5 days) [41–44]; however, serum IgGs have half-lives of ~3 weeks (Table 1.3). The prolonged half-lives of IgGs are mainly due to the protective and recycling action of the FcRn [22,45,46].

Table 1.3 Summary of the pharmacokinetics of antibody variants engineered for increased FcRn binding in nonhuman primates.

Mutation(s) (EU numbering)	IgG isotype	Target antigen	FcRn affinity increase at pH 6.0 (fold)	Serum half-life (fold of WT)	Clearance (Fold of WT)	Source
M428L	IgG2	α-HBV OST577	~7× (human)[a)] ~8× (rhesus)[a)]	1.8× (rhesus)	0.56× (rhesus)	[47]
T250Q/ M428L	IgG2	α-HBV OST577	~28× (human)[a)] ~27× (rhesus)[a)]	1.8× (rhesus)	0.36× (rhesus)	[47]
M252Y/ S254T/ T256E	IgG1	α-RSV	~11× (human)[b)] ~9× (rhesus)[b)]	3.5× (cyno)	N.A.	[48]
T250Q/ M428L	IgG1	α-HBV OST577	~29× (human)[a)] ~37× (rhesus)[a)]	2.5× (rhesus)	0.42× (rhesus)	[49]
P257I/ N434H	IgG1	TNFα	~16× (human)[c)] ~52× (cyno)[c)]	0.8×[d)] (cyno)	1.1×[d)] (cyno)	[50]
D376V/ N434H	IgG1	TNFα	~15× (human)[c)] ~52× (cyno)[c)]	0.7× (cyno)	1.3×[d)] (cyno)	[50]
T250Q/ M428L	IgG1	TNFα	~40× (cyno)[c)]	0.9× (cyno)	1.1×[d)] (cyno)	[51]
P257I/ Q311I	IgG1	TNFα	~19× (human)[c)] ~80× (cyno)[c)]	0.8× (cyno)	0.8×[d)] (cyno)	[50,51]
N434A	IgG1	Unknown	~3× (human)[b)]	1.6× (cyno)	0.54× (cyno)	[52]
N434W	IgG1	Unknown	~38× (human)[b)]	1.0×[d)] (cyno)	1.2×[d)] (cyno)	[52]

(continued)

Table 1.3 (*Continued*)

Mutation(s) (EU numbering)	IgG isotype	Target antigen	FcRn affinity increase at pH 6.0 (fold)	Serum half-life (fold of WT)	Clearance (Fold of WT)	Source
M428L/ N434S	IgG1	α-VEGF	∼11× (human)[b)]	3.2× (cyno)	0.32× (cyno)	[53]
V259I/ V308F	IgG1	α-VEGF	∼6× (human)[b)]	1.7× (cyno)	0.63× (cyno)	[53]
M252Y/ S254T/ T256E	IgG1	α-VEGF	∼7× (human)[b)]	2.5× (cyno)	0.42× (cyno)	[53]
V259I/ V308F/ M428L	IgG1	α-VEGF	∼20× (human)[b)]	2.6× (cyno)	0.39× (cyno)	[53]
M428L/ N434S	IgG1	α-EGFR	∼11× (human)[b)]	3.1× (cyno)	0.31× (cyno)	[53]
N434H	IgG1	α-VEGF	∼4× (human)[b)] ∼5× (cyno)[b)]	1.6× (cyno)	0.62× (cyno)	[54]
T307Q/ N434A	IgG1	α-VEGF	∼18× (human)[b)] ∼10× (cyno)[b)]	2.2× (cyno)	0.52× (cyno)	[54]
T307Q/ N434S	IgG1	α-VEGF	∼10× (human)[b)] ∼12× (cyno)[b)]	2.0× (cyno)	0.49× (cyno)	[54]
T307Q/ E380A/ N434A	IgG1	α-VEGF	∼13× (human)[b)] ∼15× (cyno)[b)]	1.9× (cyno)	0.57× (cyno)	[54]
V308P/ N434A	IgG1	α-VEGF	∼26× (human)[b)] ∼34× (cyno)[b)]	1.8× (cyno)	0.57× (cyno)	[54]
N434H	IgG1 (N297A)	CD4	∼3× (human)[b)] ∼3× (baboon)[b)]	N/A	0.50× (baboon)	[55]
V308P	IgG4	5 unknown targets	∼40–390× (cyno)[c)]	2.0–3.3× (cyno)	0.22–0.74× (cyno)	[56]
T250Q/ M428L	IgG4	5 unknown targets	∼11–110× (cyno)[c)]	0.9–2.6× (cyno)	0.31–0.89× (cyno)	[56]

Abbreviations: EGFR, endothelial cell growth factor receptor; FcRn, neonatal Fc receptor; HBV, hepatitis B virus; N/A: not available; RSV, respiratory syncytial virus; TNF, tumor necrosis factor; VEGF, vascular endothelial cell growth factor.

a) IC_{50} binding ratio performed on FcRn-transfected cells.

b) Monovalent interaction: injecting FcRn over surface-conjugated antibodies.

c) Bivalent interaction: injecting antibodies over surface-conjugated FcRn.

d) No statistically significant difference.

FcRn is also known as Fc receptor-protection (FcRp) or Fc receptor-Brambell (FcRB) [57] after F.W. Rogers Brambell who first described it and its protective function. In the late 1950s, Brambell proposed that a saturable receptor existed for transporting IgG from mothers to infants through the yolk sacs and intestines [57,58]. Observing the similarity between passive transmission and catabolism of IgG, Brambell later postulated that a similar or identical receptor system was responsible for the protection of IgG from catabolism [59]. It was not until 1989 that FcRn was finally cloned from the epithelial cells of the small intestine of a rat and confirmed to carry out important functions of both transporting IgG across cellular barrier (transcytosis) [58] and rescuing IgG from catalytic degradation (homeostasis) [60,61] (Figure 1.3). Even though transcytosis and homeostasis processes are both mediated by FcRn, the respective processes are regulated differently inside cells [62]. Studies have also shown that FcRn is involved in the internalization [63], presentation, and cross-presentation of immune complex onto the major histocompatibility complex (MHC) [64–66].

FcRn, which is structurally homologous to MHC class I molecules, is a heterodimer consisting of a 50 kDa transmembrane α chain and 12 kDa β2-microglobulin (β2m) (Figure 1.1, panel e), which is required for the FcRn expression. FcRn mostly resides intracellularly, but can be exposed to the extracellular environment through vesicle trafficking [9,67]. Unlike MHC molecules, the counterpart of the MHC peptide-binding groove in FcRn is occluded by its own residues, so FcRn is incapable of binding peptides and hence does not present peptide–MHC complex to T cells [9,67]. FcRn can simultaneously bind both IgG and albumin, but the binding stoichiometries are different, with a 2 : 1 ratio for FcRn–IgG and a 1 : 1 ratio for FcRn–albumin [21,68]. It was previously shown that bivalent binding of an IgG to FcRn enhances the rate of IgG recycling inside a cell [69].

Although a crystal complex structure of human FcRn–Fc is still not available, the major contact residues in the human complex can be deduced from the crystal structure of a rat FcRn–Fc complex [21,70] and site-directed mutagenesis studies [71–74]. At the protein level, FcRn binds to the Fc portion of IgG at the C_H2–qC_H3 interface, which is distinct from the binding sites of FcγR and C1q component of complement (Figure 1.1, panel e) [21,39,67,72]. It is worth noting that Fc alone can mediate binding to FcRn, so in addition to IgG, FcRn can protect Fc-fusion proteins from degradation. Molecularly, major contact residues of the human FcRn are Glu115, Asp130, Trp131, Glu133, and Leu135 on the α chain and Ile1 on the β2m. On the Fc side, residues Ile253, Ser254, His310, His435, and Tyr436 are important for the interaction, as alanine substitution at these positions results in a significant reduction in binding to FcRn [72]. Meanwhile, FcRn has been found to bind albumin around His166, opposite from the Fc binding region [75], which may explain why FcRn can simultaneously bind both IgGs and albumins [71].

FcRn protects IgGs from catabolism through a pH-dependent binding mechanism. IgGs bind FcRn with high affinity at acidic pH (pH ∼ 6–6.5). As the pH is raised to neutral (pH ∼ 7.4), the binding affinity drops considerably. The pH-dependent interaction is mainly attributed to the titration of histidine residues

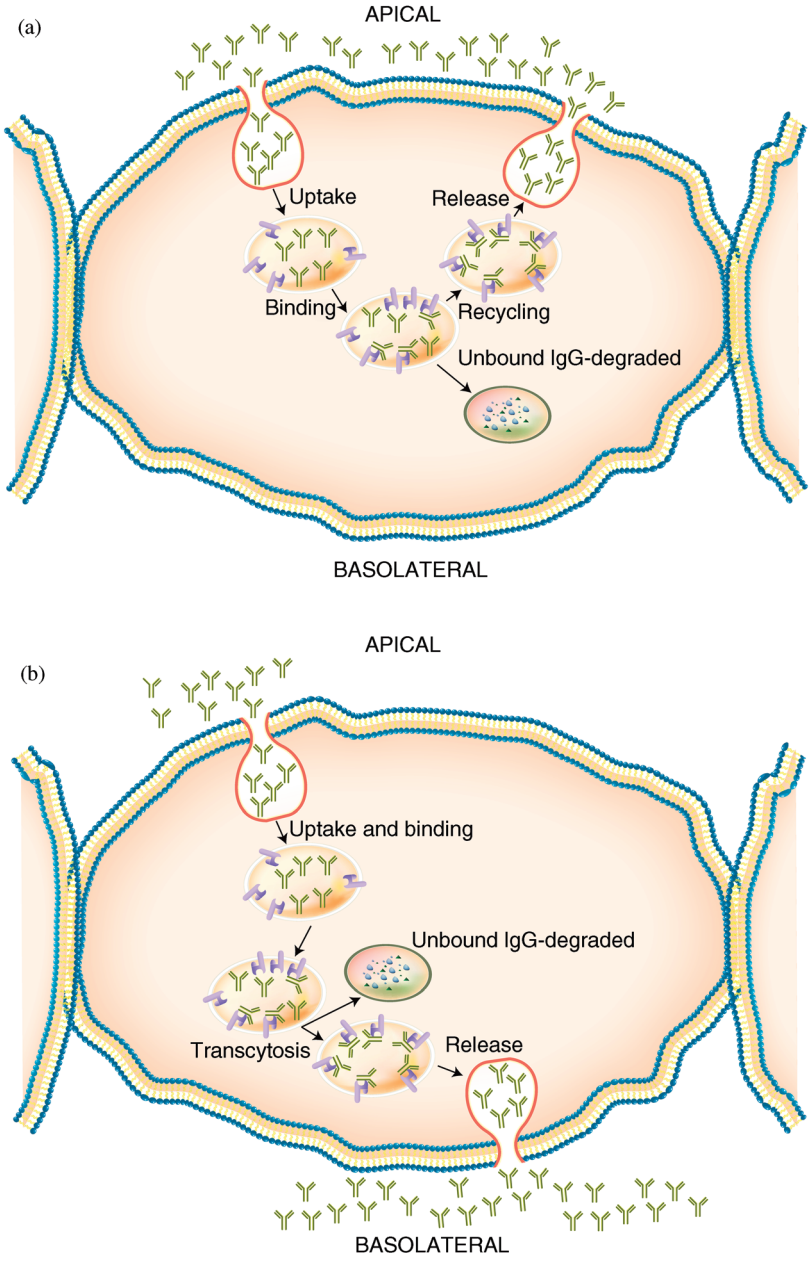

Figure 1.3 Antibody recycling. (a) Transcytosis. (b) IgGs are pinocytosed into the cell, captured by FcRn in the acidic endosome, recycled to the cell surface, and then released into the circulation at a physiological pH of 7.4. Depending on the intracellular signaling within different cell types, IgGs are either recycled to the apical side (a) or transported to the basolateral side (b). IgGs that are not bound by FcRn inside the cell are transported to the lysosome and degraded.

(H310 and H433) on human Fc and their subsequent interaction with acidic residues on FcRn [76]. Specifically, pinocytosed IgGs are captured by FcRn in the acidic endosome [77], recycled to the cell surface [78], and then released into the circulation at a physiological pH of 7.4 [79]. IgGs that are not bound by FcRn are transported to the lysosome and degraded [77]. Studies in knockout mice have demonstrated that the serum half-lives of IgGs and albumin in FcRn- or β2m-deficient mice are greatly reduced [45,60]. It has also been observed in familial hypercatabolic hypoproteinemia patients that their low levels of serum IgGs and albumin were caused by the reduction of FcRn expression, resulting from β2m deficiency [61].

Functional FcRn expression has been reported in a variety of tissues and cells such as vascular endothelium [80], hematopoietic cells (monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, and B cells) [81], intestinal epithelium [82], brain and choroids plexus endothelium [83,84], podocytes [85], placental endothelium [86,87], lung epithelium [88], and vaginal epithelium [64]. FcRn has been shown in studies to either recycle IgG or transport IgG across the cellular barriers. As FcRn is ubiquitously expressed in multiple cell types like endothelial, epithelial, and hematopoietic cells at various body sites, it is of interest to understand which cell types and tissues are responsible for the IgG recycling. First, Akilesh *et al.* [89] used bone marrow chimeric mice with FcRn-deficient and FcRn-sufficient cells to demonstrate that a significant fraction of IgG protection is mediated by hematopoietic cells. Later, by using a mouse strain in which FcRn is conditionally deleted, Montoyo *et al.* [90] were able to show that transgenic mice without FcRn expression in endothelial and/or hematopoietic cells did not protect IgGs from degradation, indicating that both hematopoietic cells and endothelial cells are the primary sites for maintaining IgG homeostasis in mice.

1.3.2 Species Difference in FcRn

FcRns of multiple mammalian species have been cloned [91], and functional expression of FcRn has been reported in mammals like the rat, mouse, rabbit, sheep, bovine, possum, horse, pig, nonhuman primate, and human [57,92–95]. An FcRn orthologous molecule has also been described in chicken [96]. In terms of sequence homology of FcRn in difference species, primate FcRn is the closest to human FcRn. For example, cynomolgus monkey FcRn has a 96% sequence identity (98% similarity) to human FcRn and the two receptors bind human IgG with similar affinity. Meanwhile, rat and mouse FcRns are 91% identical but have only 69% identity (80% similarity) and 70% identity (80% similarity) with human FcRn, respectively. Molecularly, rodent FcRn contains four N-linked glycans while human FcRn has only one N-linked glycan [73]. In rodent FcRn, carbohydrate residues in the glycan at Asn 128 participate in binding mouse Fc [73]. Along with glycan differences, the amino acid differences between rodent and human FcRns give rise to different cross-binding specificity and affinity between rodent and human FcRn–Fc [74]. For example, mouse FcRn, which is considered the most

promiscuous, was able to bind IgGs of human, rabbit, bovine, rat, sheep, and guinea pig, whereas human FcRn was unable to bind mouse and rat IgGs, showing restricted binding to only human and rabbit IgGs [97]. In addition, human IgGs have higher affinity toward murine FcRn than murine IgG [98]. Because of the binding affinity and specificity differences, it has been challenging to use rodent models to evaluate PK of human IgG Fc variants that were engineered for human FcRn binding (see more detailed discussion in Section 1.3.3) [48].

1.3.3 Engineering to Modulate Pharmacokinetics

1.3.3.1 Fc Engineering

As FcRn interaction is responsible for the PK of IgG and Fc-fusion therapeutics, engineering the FcRn–Fc interaction is one of the methods for modifying the PK and pharmacodynamics (PD) of an IgG or Fc fusion [22,99]. The one area that is most researched and attractive to the pharmaceutical industry is the attempt to increase the serum half-life of the therapeutic IgG and Fc fusions. Advantages of increased serum half-life include increasing transcytosis to maximize drug delivery at specific tissues, minimizing adverse reactions caused by high doses, decreasing production cost, and reducing frequency of injection, thereby potentially increasing the compliance of patients taking the drugs. A number of studies have demonstrated that balancing the affinity/kinetic improvements of Fc variants at pH 6.0 and pH 7.4 is critical for engineering variants with improved half-life. Increasing affinity at pH 6.0 can increase the capture of IgG variants by FcRn in the endosome, thereby reducing degradation and increasing the recycling chances. However, affinity increases of variants at pH 6.0 and pH 7.4 are often coupled, so a substantial increase in a variant's FcRn affinity at pH 6.0 will lead to an undesirable increase in affinity at pH 7.4 [50,51]. High levels of binding at pH 7.4 hinder the release of an FcRn-bound IgG variant back into circulation and increase the binding of circulating IgGs to the cell surface-expressing FcRn, effectively accelerating the clearance of IgG and canceling out the benefit of increased affinity at acidic pH. Therefore, FcRn binding affinities/kinetics at both acidic and physiologic pHs are important determinants to balance the PK engineering of an IgG or Fc fusion.

The first proof-of-concept study of engineering Fc for improved half-life was performed by Ghetie *et al.* [100], who observed that an engineered murine Fc variant (T252L/T254S/T254F) with a threefold increase in murine FcRn affinity at pH 6.0 exhibited approximately a 30–60% extension in serum half-life in mice. Subsequent studies have identified and validated various favorable mutations on human IgG1 Fc residues, such as Thr250, Met252, Thr254, Ser256, Thr307, Val308, Glu380, Met428, and Asn434 [49,54,56,72,101]. These mutations can be combined synergistically to give Fc variants of different affinities, yielding half-life improvements of varying extents (Table 1.3).

One important aspect of engineering the half-lives of IgGs is the use of suitable preclinical animal models to evaluate the variant's half-life *in vivo* and predict the

variant's PK parameters in humans. Earlier studies involved testing human IgG1 Fc variants in a mouse model; however, because of the affinity and specificity differences in human IgGs binding to murine and human FcRn [102], PK results of the variants in mice did not truly reflect the variant's PK in humans [48]. This has led to development of human FcRn transgenic mice, which lack endogenous murine FcRn, but express human FcRn [101]. PK differences between human IgG Fc variants carrying human FcRn-specific mutations with large affinity differences can be distinguished in these transgenic mice [52,101]. However, further developments, including control of FcRn expression levels and patterns [103] and endogenous IgG levels, are needed to render these transgenic mice more humanlike in order to distinguish fine PK differences between variants with little affinity changes. To date, despite the high cost, the most relevant, physiologically accurate model system for human is still nonhuman primates as they have similar levels of endogenous IgG, and human IgG1 binds nonhuman primate FcRn with similar affinity and specificity as human FcRn [47–49].

An IgG can also be engineered to have a shorter half-life *in vivo* by reducing its FcRn affinity. It has been shown that an Fc variant's half-life is reduced in correspondence with decreasing FcRn affinity [99,104]. Short-lived IgG Fc variants can be useful in situations where shorter IgG exposure is desired, for example, imaging, radioimmunotherapy, and toxin-conjugated antibody therapy. It is also worth noting that an IgG can be engineered to have high neutral pH binding to FcRn [105]. Such IgG variants would have short serum half-lives, but high doses of such variants can block FcRn function, thereby reducing the half-lives of endogenous IgG. This is potentially useful in promoting the clearance of endogenous pathogenic antibodies in autoimmune patients.

To date, PK-modifying IgG Fc mutations are being incorporated into therapeutic IgGs or Fc fusions, and more of these variants are being evaluated in clinical trials. One recently published trial evaluated the PK of a human anti-CD4 IgG1 (N297A) Fc variant (N434H) in patients with rheumatoid arthritis [55]. When comparing results from two different cross-studies, PK of N434H in the diseased population was not improved over wild type in the normal healthy population; however, the PK result for N434H was confounded by pre-existing rheumatoid factors against the N434H mutation, which in turn may have affected the PK of the variant. Therefore, the study could not definitively conclude how the FcRn mutation (N434H) impacted the variant's half-life in humans. Overall, Fc engineering appears to be a promising way to modulate the PK of an antibody or Fc fusion, but it remains to be seen how much PK improvement and immunogenicity of these Fc variants will be achieved in humans.

1.3.3.2 Other Engineering Efforts to Modify PK of an IgG or Fc Fusion

In addition to the nonspecific clearance mechanism, target-dependent clearance is another major route where antibody is eliminated from the circulation. Typically, antibodies targeting antigens that show significant antigen-mediated clearance have reduced serum half-life and hence high doses and/or frequent dosing is required. Examples of such antigens are cell receptors (e.g., IL6R, EGFR, CD4, and

CD40) and abundantly synthesized soluble antigens like PCSK9 and IgE. In addition to studies that have shown that engineering optimized FcRn binding can extend the serum half-life of antibodies [53], other work has demonstrated that the half-life of some antibodies can be extended by engineering their interactions with antigens for increased pH dependency [106–108]. Such pH-sensitive antibodies would bind tightly to the receptor/antigen in the plasma (pH 7.4); once trafficked inside cells, the antibody–antigen complex would dissociate in the acidic endosome and the antibody would recycle back into the circulation by FcRn, effectively avoiding lysosomal degradation and allowing the same antibody to undergo further binding cycles. Such pH-engineered antibodies were shown to have improved half-lives *in vivo* compared to their parental counterparts, particularly at lower doses [109,110].

Besides Fc–FcRn and antibody–antigen interactions, other biochemical factors affect the PK of an antibody, such as molecular size, molecular charges, stability of the IgG, and glycosylation patterns, along with route of delivery [111]. A recent study suggested that engineering the isoelectric point (pI) of the antibody variable region could offer an alternative way to improve PK [107]. The typical pI of an antibody is 8–9. It has been shown that antibodies with higher pI values tend to have faster clearance *in vivo* [95]. This reduced PK was hypothesized to be due to the unfavorable electrostatic interaction between the anionic cell membrane and the positive charge of an antibody. Igawa *et al.* [107] engineered variants with pI values 1–2 units lower than the wild type and showed that these pI variants displayed longer half-lives and reduced clearance in mice. This pI-lowering effect can also be achieved using conjugation of charge moieties [112].

Overall, there are multiple methods for engineering the PK of an antibody. However, each antibody and its target antigen have very different biochemical, biophysical, and cellular properties on which antibody PK is highly dependent. Therefore, a one-size-fits-all engineering method to improve the PK of every antibody may not exist. Choosing the appropriate PK engineering method depends on understanding the properties and interactions between an antibody and its antigen, and the desired antibody exposure and distribution levels.

1.4 Introduction to FcγR- and Complement-Mediated Effector Functions

Antibodies are multifunctional molecules resulting from adaptive immunity. Part of the variable region binds to the target antigen and different parts of the constant region are responsible for diverse effector functions. This enables the antibody to bridge between the target antigen and the body's immune system. The antibody is able to recruit cellular and noncellular immune responses, which interact with each other in a complex way. In this chapter, the effector functions leading to cell lysis, phagocytosis, immune activation, and T-cell activation properties, which are essential for therapeutic antibodies against tumor cells and for infectious diseases, will be discussed.

1.4.1 Cell Lysis and Phagocytosis Mediation

Cell lysis can be mediated in two ways: (1) ADC mediated by FcγR-expressing NK cells. Immune complex-mediated activation of the NK cells by FcγR results in cytokine release such as interferon-γ (IFNγ) and cytotoxic granules. These perforin- and granzyme-containing granules enter the target cell and induce cell death through apoptosis. ADCC has also been induced by monocytes and eosinophils [113]. (2) CDC is initiated when C1q, the initiating component of the classical complement pathway, is fixed to the Fc portion of target-bound antibodies. Once C1q binds to the antibody Fc, the complement cascade is activated and leads to a membrane-spanning, multiprotein pore complex. This pore is called the membrane attack complex (MAC) and can lyse the cells (for more detailed information, see Ref. [114]).

Phagocytosis can be mediated in two ways: (1) Antibody dependent cellular phagocytosis (ADCP) is mediated by FcγR-expressing monocytes/macrophages, neutrophils, and dendritic cells (DCs). After activation of the phagocytes by FcγR, the antibody-coated cell is engulfed and degraded once the phagosome fuses to the lysosome [115]. (2) Complement-dependent cellular cytotoxicity (CDCC) can be initiated when opsonic membrane-bound components (C3b, iC3b, C4b) of the complement cascade interact with complement receptors (CR1, CR3, CR4, CRIg) on immune cells. This interaction can result in phagocytosis by monocytes/macrophages, neutrophils, and DCs, while cell lysis is mediated by NK cells [114]. CDCC only occurs as a response to yeast- and fungi-produced proteins because CR3 requires dual ligation to iC3b and the cell wall β-glucan, which is only found on yeast or fungi [116].

1.4.2 FcγR-Mediated Effector Functions

1.4.2.1 FcγR Biology

Antibodies interact with effector cells via binding of their Fc region to cellular FcγR. In humans, six known members in three subgroups of FcγR (FcγRI, FcγRIIabc, FcγRIIIab) exist, with differences in expression, signaling, and affinities for the IgG subclasses.

Four activating receptors with cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) exist, which are genetically encoded or associated. The inhibitory FcγRIIb contains an immunoreceptor tyrosine-based inhibition motif (ITIM), which is genetically encoded in the same molecule. The glycosyl phosphatidyl inositol (GPI)-anchored FcγRIIIb does not signal. ITAM/ITIM signaling is based on receptor clustering induced by binding to immune complexes. No clustering occurs for FcγRII/III binding to monomeric IgG because of the low affinity. The high affinity of FcγRI can poorly differentiate between monomeric IgG and immune complexes. FcγRIIa and FcγRIIIa have allelic forms, which can impact their affinity to different IgG subclasses (Table 1.4) and ultimately their biological function. FcγRIIa contains a polymorphism at position

Table 1.4 Affinity of human IgG subclasses for human and mouse FcγRs and the expression profile of FcγRs.

Human							
Receptor/ allele	**hFcγRI**	**hFcγRIIa/ 131H**	**hFcγRIIa/ 131R**	**hFcγRIIb**	**hFcγRIIc**	**hFcγRIIIa/ 158F**	**hFcγRIIIa/ 158V**
Gene	CD64	CD32a	CD32a	CD32b	CD32c	CD16	CD16
Cytoplasmic	ITAM, associated	ITAM, genetic	ITAM, genetic	ITIM, genetic	ITAM, genetic	ITAM, associated	ITAM, associated
Affinity (K_D, $\times 10^{-6}$ M) [117]							
hIgG1	0.02	0.19	0.29	8.33	0.85	0.50	5.00
hIgG2	nb	2.22	10.00	50.00	33.33	14.00	nb
hIgG3	0.02	1.12	1.10	5.88	0.13	0.10	0.91
hIgG4	0.03	5.88	4.76	5.00	50.00	4.00	nb
Expression	MΦ	MΦ	MΦ	MΦ	MΦ?	MΦ	MΦ
	DC	DC	DC	DC	NE	DC	DC
	PMN	PMN	PMN	PMN	PMN?	PMN	PMN
					NK[a)]	NK	NK
Murine functional homologues[b)]							
Receptor	**mFcγRI**	**mFcγRIV**		**mFcγRIIb**		**mFcγRIII**	
Gene	CD64	CD16-2		CD32B		CD16	
Cytoplasmic	ITAM, associated	ITAM, associated		ITIM, genetic		ITAM, associated	
Affinity (K_D, $\times 10^{-6}$ M) [118]							
hIgG1	0.26	0.46		2.00		28.57	
hIgG2	nb	nb		>100		>100	
hIgG3	0.83	100		12.05		nb	
hIgG4	13.89	>100		>100		>100	
Expression	MΦ	MΦ		MΦ		MΦ	
	DC	DC		DC		DC	
		PMN		PMN		PMN	
						NK	

Abbreviations: ITAM, tyrosine-based activation motif; ITIM, tyrosine-based inhibition motif; nb, no detectable binding; MΦ, macrophages; DC, dendritic cells; NE, no expression; PMN, polymorphonuclear neutrophils; NK, natural killer cells.

a) Expressed in only 30% of humans.

b) Murine functional homologues are based on similarity of expression patterns, not on sequence homology.

131 (histidine and arginine). Only histidine has high enough affinity to carry out IgG2-mediated phagocytosis by neutrophils and monocytes. IgG1 and IgG3 bind with high affinity to the valine 158 and with lower affinity to phenylalanine of FcγRIIIa (Table 1.4).

1.4.2.2 Expression Profiles

A variety of immune cells express FcγRs, including NK cells, monocytes/ macrophages, DCs, neutrophils, basophils, eosinophils, mast cells, B cells, and γδ

T cells. The most relevant for FcγR-induced effector functions of therapeutic antibodies are NK cells, monocytes/macrophages, DCs, and neutrophils.

NK cells are unique because in most people they typically only express the activating receptor FcγRIIIa while NK cells in some individuals express the activating FcγRIIc receptor. The main FcγR-induced functions of NK cells are the cytolysis of target cells through lytic granule release (granzyme, perforin), apoptosis via secretion of tumor necrosis factor (TNF) family ligands, and production of cytokines such as IFNγ. A series of activating receptors, like NKG2D, and inhibitory receptors of the killer Ig-like receptor (KIR) family regulate the NK cell activity. On normal cells, the killing is suppressed because the KIRs interact with autologous MHC class I molecules. Killing is induced if matching MHC molecules are missing. Antibody-coated target cells can be killed by FcγRIIIa engagement because KIR inhibition is partially overridden.

Monocytes/macrophages, neutrophils, and DCs (myeloid cell lineage) have overlapping FcγR expression profiles and all of them express FcγRIIa and FcγRIIb. FcγRI and FcγRIIIa are also expressed depending on their source and activation state by monocytes/macrophages and DCs; for example, after G-CSF activation neutrophils express FcγRIIIb rather than FcγRI and FcγRIIIa.

Upon FcγR engagement, macrophages and neutrophils can phagocytose opsonized target cells. They lyse target cells by releasing cytolytic granules or inducing apoptosis via release of reactive nitrogen and oxygen intermediates. Besides target-cell destruction, macrophages and DCs are also professional antigen-presenting cells and can present peptides of target cell antigens on MHC class II to $CD4^+$ T cells. DCs can additionally present peptides of target antigens on MHC class I and activate cytotoxic T cells ($CD8^+$) by cross-priming, which can lead to long-lasting adaptive antitumor immunity and long-term remission. This was observed for the anti-CD20 antibody rituximab [119].

1.4.2.3 Therapeutic Relevancy

The most compelling data are obtained from associations of clinical outcomes with functionally relevant receptor polymorphisms. FcγR polymorphisms have been associated with infectious and autoimmune disease, or with disease severity [120,121]. In humans, the FcγRIIa-H131 allotype is known to interact efficiently with complex human IgG2, whereas the FcγRIIa-R131 allotype does so only poorly. This polymorphism may therefore have implications for IgG2-mediated phagocytosis of encapsulated bacteria and susceptibility to bacterial infections. FcγRIIa-R131 is associated with greater susceptibility to infectious diseases [120,122]. Polymorphism association studies have been applied to cancer therapies using monoclonal antibodies [115]. Significant response differences between high-affinity V158 and low-affinity F158 FcγRIIIa alleles have been observed with rituximab (anti-CD20) for the treatment of follicular non-Hodgkin's lymphoma [123], Waldenström's macroglobulinemia [124], and in two out of three studies in diffuse large B-cell lymphoma [125–127]. No FcγR polymorphism was observed for rituximab treatment in chronic lymphocytic leukemia (CLL) [128,129]. Apart from the clinical relevancy of FcγRIIa for infectious diseases [120,122], the clinical outcome of murine IgG3 anti-GD2 antibody 3F8 in the solid tumor neuroblastoma

is correlated with FcγRIIa-R131 [130]. Murine IgG3 has preferential binding of human FcγRIIa-R131 over H131, which are only expressed on neutrophils, macrophages, and DCs, while NK cells do not express FcγRIIa. In a following clinical study, 3F8 was administered in combination with granulocyte macrophage colony-stimulating factor (GM-CSF) and suggested that granulocytes (e.g., neutrophils) are relevant effector cells [131]. Future studies will perhaps bring a clearer picture of the importance of activating FcγR since many ADCC-enhanced therapeutic antibodies are in the clinic [132,133].

1.4.3 Complement

1.4.3.1 C1q Biology

The lectin pathway and alternative pathways are generally activated by pathogens and not by cell-bound antibodies and are therefore not further discussed. The classical pathway of the complement system can be activated following binding of monoclonal antibodies to tumor cells. This pathway can be initiated on binding of the C1q component of the C1 complex to the Fc of the antibody on the cell membrane. The initiation of the pathway by the antibody is dependent on the subclass of bound antibody (IgG3 > IgG1 ≫ IgG2 > IgG4), the membrane proximity of the antibody epitope, the membrane protein number per cell, and the affinity of the antibody, which leads to simultaneous binding of one C1 complex to at least two Fcs [116,134]. The initiation of the pathway results in the deposition of C3b, which is subsequently converted to iC3b and can lead to the formation of the cytolytic MAC. This complex results in CDC, which is a noncellular activity. The initiation of the pathway by antibodies also leads to the deposition of opsonic proteins (C3b, iC3b, and C4b) on the cell surface, which can lead to two cellular complement activities. First, cell surface-bound opsonins can bind the complement receptors (CR1, CR3, CR4, and CRIg) on phagocytes and NK cells, which can trigger CDCC. This only occurs in cases where the cell wall β-glucan from yeast or fungi is present. Therefore, tumor cells do not trigger CDCC. Second, opsonin–CR interaction and C5a trigger the enhancement of ADCC. C5a function is a chemoattractant for effector cells and lowers the threshold for FcγR activation by upregulating the expression of activating FcγR and downregulating the inhibitory FcγR. To prevent uncontrolled activation and consumption of complement components, complement activation is tightly regulated by complement regulatory proteins (CRPs). CRPs are present as soluble proteins and as membrane-bound complement regulatory proteins on most cell types [114,116].

1.4.3.2 Therapeutic Relevancy

The importance of the complement system for antibodies against infectious diseases has been shown in mouse models [135]. The role of the complement system for anticancer antibodies is not well understood and may be even detrimental. Data from mouse models and clinical trials are contradictory [113,136]. The complement activation of anti-CD20 antibodies was found to correlate with

infusion toxicity of rituximab [137]. The best support comes from enhanced activity of a CDC-enhanced anti-CD20 antibody in nonhuman primates [138] and from the approval of ofatumumab, a CDC-enhanced anti-CD20 antibody [139].

1.4.4 Modifying Effector Functions

1.4.4.1 FcγR-Dependent Effector Function

Fc mutations with increased effector function are shown in Table 1.5. Effector cells can express several activating and inhibiting FcγR. This complicates the question of what an ideal FcγR selectivity profile for increasing ADCC and ADCP of an

Table 1.5 Fc mutations with increased effector function.

Mutation	Isotype	ADCC	ADCP	CDC	Source
S298A, E333A, K334A	hIgG1	↑	↑		[72]
S239D, I332E	hIgG1	↑	↑		[147–149]
S239D, A330L, I332E	hIgG1	↑	↑	↓	[147,150]
H268F/S324T	hIgG1	↔	↔	↑	[151]
S267E/H268F/S324T	hIgG1	↓	↔	↑	
S239D/I332E/H268F/S324T	hIgG1	↑	↑	↑	
S236A/I332E/H268F/S324T	hIgG1	↑	↑	↑	
S267E/S236A/I332E/H268F/S324T	hIgG1	↔	↓	↑	
G236A/I332E	hIgG1	↑	↑	↔	
P247I, A339(D/Q)	hIgG1	↑	↑		[152]
D280H, K290S or D280H, K290S, [S298 (D/V)]	hIgG1	↑	↑		[153]
F243L, R292P, Y300L or F243L, R292P, Y300L, P396L or F243L, R292P, Y300L, V305I	hIgG1	↑	↑		[154]
G236A, S239D, I332E	hIgG1		↑		[141]
K326A, E333A	hIgG1	↔		↑	[155]
K326W, E333S	hIgG1	↓		↑	
E333S (K326W)	hIgG2			↑	
F243L[a)]	hIgG1	↑			[156]
"1133" "113F'"	hIgG1/3 fusion	↔		↑	[138]
K290(E/N), S298G, T299A, and/or K326E	Aglyc. hIgG1	↑[b)]			[157]
E382V, M428I	Aglyc. hIgG1	↑[c)]			[158]
Afucosylated (AF)	hIgG1	↑	↑		[159]
S298A, K326A, E333A, K334A	hIgG1	↑	↑		
S298A, K326A, E333A, K334A-LF	hIgG1	↑	↑		

Abbreviations: AF, afucosylated; LF, low fucosylated; aglyc, aglycosylated; ↑, increase; ↓, decrease; ↔, maintained.

a) Affects glycosylation.

b) Improved FcγRI binding, similar FcγRIIa/b binding.

c) Selective binding to FcγRI with nanomolar affinity (no effect on FcRn binding).

engineered antibody should look like. The activating FcγRI does not seem very important, considering that the high affinity for monomeric IgG results in poor capability to distinguish between monomeric and immune complexes. Additionally, FcγR$^{-/-}$ mice did not have an impact on antibody activity [140]. The importance of FcγRIIIa is well established since the clinical outcome of rituximab correlates with high-affinity FcγRIIIa-158V allele and FcγRIIIa is the only FcγR displayed on NK cells. The importance of FcγRIIa was shown in the clinical outcome of the murine IgG3 anti-GD2 antibody 3F8 and through the dominant role for macrophages [141]. The importance of the inhibitory FcγRIIb of therapeutic antibodies in mice was increased in FcγRIIb$^{-/-}$ mice over wild-type mice [142,143]. The selective blockage of FcγRIIb on DCs with an antagonistic anti-FcγRIIb antibody in the presence of IgG in human plasma resulted in spontaneous activation [144]. In macrophages, FcγRIIb engagement has been shown to downregulate phagocytosis and cytokine release [145]. Altering the ratio of affinities between activating over inhibiting receptor (A/I ratio) of antibodies could allow greater control over the activation of certain cell types [146]. Engineering an increased A/I ratio for FcγRIIa/FcγRIIb is very difficult since the extracellular domains are 96% identical, and increasing the FcγRIIIa/FcγRIIb ratios will most probably lead to simultaneously decreased affinity to FcγRIIa. The *in vitro* comparison of FcγR-selective mutations on the phagocytosis of macrophages demonstrated the dominant role of the absolute affinity for FcγRIIa over FcγRIIa and FcγRI. Interestingly, FcγRIIb did not show an impact [141].

1.4.4.2 Engineering

Choosing the appropriate IgG subclass will only provide one with nature's solution; therefore, the introduction of mutations into human IgG1 has been used to increase IgG effector functions based on published FcγR/Fc crystal structures. Several groups obtained mutations for increased binding to FcγRIIIa/FcγRIIa/FcγRIIb, which increased the affinity to FcγRIIIa between one and two orders of magnitude [72,147,154]. The increased ADCC potency and efficacy *in vivo* was shown in human FcγRIIIa-transgenic mice and in a monkey B-cell depletion model [147,149,154]. Several engineered antibodies with these mutations have entered clinical trials [132].

Antibodies with increased FcγRIIIa/FcγRIIb ratios were also obtained, but the effector function benefits based on the increased FcγRIIIa/FcγRIIb ratios are still not clear [113]. Mutations with up to 70-fold increased affinity to FcγRIIa resulted in enhancement of macrophage phagocytosis, while improvement of FcγRIIa/FcγRIIb demonstrated the dominant role of the absolute affinity to FcRIIa [141]. Antibodies that bind only to FcγRI were obtained by engineering aglycosylated Fc fragments [158].

1.4.4.3 Glycoengineering

In Section 1.2.1.1, the glycosylation patterns of Fc were described. More than 80% of Fc glycans in serum IgG and more than 90% of recombinant IgGs produced in

normal CHO cells are fucosylated. Nonfucosylated IgGs showed more than 50-fold increased ADCC *in vitro* [37]. Nonfucosylated antibodies increased affinity for the two polymorphic forms of soluble human FcγRIIIa by up to 50-fold while the affinities for FcγRI and C1q were not increased, and the affinities for FcγRIIa and FcγRIIb were only very moderately increased or not increased at all [36,160,161]. The crystal structures between FcγRIIIa and fucosylated and nonfucosylated Fcs were recently published [29,162]. Furthermore, it was shown that the amount of nonfucosylated glycan in antibody samples correlates in a linear fashion with FcγRIIIa binding and ADCC activity [161]. For the production of nonfucosylated antibodies, different strategies have been used [163]. Because nonfucosylated glycans are present in endogenous-expressed IgGs, the immunogenic potential of nonfucosylated glycans should be low. Several nonfucosylated antibodies are in the clinic and recently mogamulizumab, the first glycoengineered antibody, has been approved in Japan for patients with relapsed or refractory CCR4-positive adult T-cell leukemia–lymphoma [133].

The additive effect between mutations and nonfucosylation to further increase ADCC was published [36] while others suggest that enhancing FcγRIIIa affinity beyond a certain threshold does not result in a further increase of NK-mediated ADCC [164].

The structure of the Fc–C1q complex is not available, but the interaction sites (234, 235, 270, 322, 326, 329, 331, 333) were mapped by mutagenesis and revealed that the hinge and the Fc were important [113]. The C1q binding site is distinct but overlapping with the FcγR binding site, which is important for Fc engineering. Increased C1q binding was obtained by mutations in the C_H2 [155] or hinge region [165]. A chimeric IgG1/3 was generated, which combined the higher CDC activity of hIgG3 with the higher ADCC activity of hIgG1. The enhanced cytotoxicity of a variant was confirmed in a cynomolgus monkey model using an anti-CD20 antibody [138].

1.4.4.4 Reducing and Silencing Effector Function

For many applications of therapeutic antibodies, Fc-mediated effector functions (e.g., ADCC, ADCP, and CDC) are not part of the mechanism of action. Examples of these applications include antagonistic ligand and receptor binding without cell lysis of the target cells. These Fc-mediated effector functions can be detrimental and potentially pose a safety risk by causing off-mechanism toxicity as shown in these examples: (1) muromonab-CD3 (Orthoclone OKT3), the first antibody marketed as a therapeutic, is a murine IgG2a antibody that induces both antidrug immunogenicity and a cytokine storm due to Fc–FcγR interaction [166]; (2) keliximab, a Primatized™ IgG1 anti-CD4 antibody, was developed to block the interaction between MHC peptide and T-cell receptor complex (TCR) in order to tolerize T cells or turn them anergic. Because of the human IgG1, keliximab induced substantial $CD4^+$ T-cell depletion in human clinical trials [167]; and (3) complement-mediated activity has been linked with injection site reactions [137,168]. hIgG2 and hIgG4 are not completely absent in Fc-mediated effects (Table 1.1); therefore, similar to the approach described in Table 1.5, mutations have also been introduced to reduce or silence Fc functionalities.

Table 1.6 Fc mutations that silence effector function.

Mutation	Isotype	ADCC	ADCP	CDC	FcγRIIb	Sources
L234A, L235A	hIgG1	↓	↓			[166,174]
C220S, C226S, C229S, P238S	hIgG1	↓	↓			[175]
C226S, C229S, E233P, L234V, L235A	hIgG1	↓	↓	↓		[176]
G236R,L328R	hIgG1	↓	↓			[148]
L234F, L235E, P331S	hIgG1	↓	↓	↓		[177]
N297A (aglycosylated)	hIgG1	↓	↓			[178]
Aglycosylated	hIgG1	↓	↓			[178,179]
V234A, G237A	hIgG2	↓	↓			[180]
H268Q, V309L, A330S, P331S	hIgG2	↓	↓	↓		[170]
A330S/P331S	hIgG2			↓		[169,181,182]
IgG2: 118–260; IgG4 261–447	IgG2/4 fusion	↓	↓	↓		[171,183]
V234A, L235A	hIgG4	↓	↓			[174]
L235A, G237A, E318A	hIgG4	↓	↓			[184]
S228P[a)], L236E	hIgG4	↓	↓			[173]

Abbreviations: ↓, decrease; ↑, increase.
a) Hinge stabilizing mutation.

Several hIgG1, hIgG2, and hIgG4 variants to reduce effector functions are shown in Table 1.6. Others tried to achieve the same by introducing hIgG4 residues into hIgG2 or by generating hIgG2/4 chimeras to minimize the creation of new T-cell epitopes and therefore reduce the probability of immunogenicity [169–171]. It should be noted that IgG4 are dynamic molecules that exchange Fab arms by swapping a heavy chain and attached light chain (half-molecule) with a heavy–light chain pair from another molecule [172]. This unwanted exchange behavior can be prevented by simultaneously introducing the hinge-stabilizing mutations S228P [173].

Aglycosylated Fc reduces the binding affinity to FcγRI by more than two orders of magnitude and completely abolishes binding to the weaker affinity FcγRII and FcγRIII [158]. The binding affinity for C1q is reduced by 10-fold [185]. The reduced binding affinity abrogates both ADCC and CDC. Removal of the N297 glycan can be achieved by mutation [178] or expression in prokaryotic hosts [186].

The best-studied antibody with detrimental Fc-related activities is the cytokine storm-inducing anti-CD3 antibody. Phase I clinical trials with the aglycosylated N297A IgG1 [178,187], L234A/L235A of IgG1 and IgG4 [174,188], and V234A/G237A of IgG2 [180] demonstrated that the cytokine storm was reduced successfully while maintaining immunosuppressive activity. IgG4 variants were used for the above-mentioned anti-CD4 antibody clenoliximab in order to remove unwanted T-cell depletion and the anti-CD52 monoclonal antibody (mAb) alemtuzumab [184]. IgG2 variants were used for anti-PCSK9 antibodies [189], anti-Dickkopf-1 (DKK1) [190], and anti-amyloid beta and anti-IL-13 receptor antibodies [191]. Hayden-Ledbetter *et al.* [168] used an Fc variant of hIgG1 in an attempt

to reduce injection site reactions that were thought to be amplified by complement-mediated activity while maintaining FcγR binding for B-cell depletion. Introducing A330L into FcγR-enhanced antibodies abrogated C1q binding, which could be used to reduce complement-mediated injection site reactions while keeping high ADCC activity [147].

1.5 Current Trends in Antibody Engineering

1.5.1 Bispecific

Natural antibodies are bifunctional and monospecific, generally binding two molecules of the same epitope (with the exception of IgG4 resulting from half-molecule exchange [172]). To expand the use of antibodies to modify disease, several approaches to obtain bi/multispecificity have been developed. Some of the approaches maintain the original bifunctionality while in other cases tetra- or higher functionality is obtained.

Multispecificity can be obtained by engineering more than one specificity in a single VH–VL pair (dual-acting Fab [192]), by chemically conjugating peptides of different specificity to a catalytic antibody (CovX-body [193]), by genetically linking two or several VH–VL pairs (sometimes to an Fc [194] or a VL [195]), or by converting the natural homodimeric nature of an Fc to a heterodimer (for a recent review, see Refs [196,197]).

Genetic fusion of scFv or other proteins to a homodimeric Fc (like a natural antibody) produces symmetric molecules with a valence of 2 × (number of specificities/Fc). For example, fusing an scFv of different specificity to the C-terminus of the HC of an antibody will produce a molecule that is bifunctional and tetravalent (bivalent for each functionality).

When different VL–VH pairs of fusion proteins are genetically linked to heterodimeric Fc, asymmetric multispecific Fc fusions are obtained. In the most conservative case, when two different specificity LC–HC pairs are joined in one antibody by a heterodimeric Fc, a bifunctional, bispecific (monofunctional for each specificity) molecule is obtained. This molecule closely resembles a natural antibody in molecular weight, geometry, and biophysical/biological behavior, but is capable of binding once to two different targets [198,199]. When a heterodimeric Fc is combined with genetic fusions of several fusion proteins, a multispecific, asymmetric, and complex molecule can be obtained.

Heterodimeric Fc can be obtained by several methods. Early heterodimeric Fcs were obtained by taking advantage of the heterodimerization preference of mouse IgG2a and rat IgG2b when expressed in the same cell, for example, a quadroma cell (a rat and a mouse hybridoma cell fused) [200]. Quadroma antibodies (e.g., catuximab), when used in the clinic, are very immunogenic and not suitable for most human applications. The first method to obtain human heterodimeric Fc proteins in an efficient way was designed by Carter [198] at Genentech, Inc. Their “knobs-into-holes” approach consists

of favoring asymmetric C_H3 dimers by introducing a bulky residue in one C_H3 side and accommodating this bulky side chain in the other C_H3 side by creating a hole through substitution of larger amino acids by smaller. Recently, similar approaches based on ionic interactions or a combination of hydrophobic and ionic interactions have been developed at Chugai Pharmaceutical Co., Ltd. [201], Amgen, Inc., [202], and Rinat-Pfizer, Inc. [199].

Antibody-based bispecific molecules are being used for very diverse biological applications. Conceptually, the goal is to achieve the following: (1) Obtain additive/synergistic functionality of modulating two targets; examples include (a) two receptors (EGFR + IGF-1R [203]); (b) two ligands (VEGF + Ang2 [204]); (c) one receptor and one ligand (PDGFb + VEGF [205]); and (d) two epitopes in the same target (binding two epitopes in IGFR and blocking interaction with IGF-1 and IGF-2 [203]). (2) Increase specificity; one example is bispecific antibody–drug conjugates (ADCs) where the expression of both targets in cancer cells, and just one in normal cells, allows higher therapeutic index (efficacy/toxicity) (HER2 + EpCAM [206]). (3) Mimic the interaction between the targets by acting as a bridge; examples are found in simulation of the immunological synapse by recruiting CD3-positive T cells to cancer cells with an anti-CD3/antitumor cell surface antigen bispecific antibody (CD3 + EpCAM [207]).

1.5.2 Drug Conjugates

Clinical success of bevacizumab (Avastin®; anti-vascular endothelial growth factor [208]) and trastuzumab (Herceptin®; anti-human epidermal growth factor receptor 2 (Her2)) initiated great interest in mAbs to treat multiple oncology conditions. Several mAbs have been approved, and many are progressing through clinical development [209]. Information from the large number of molecules arriving to the clinic is showing that mAbs are generally very well tolerated but are often not very efficacious. On the other hand, chemotherapy approaches have shown good efficacy killing cancer cells, but dosing limitations due to side effects have precluded the realization of maximal clinical utility. The combination of specificity and toxicity to create a "magic bullet" was already proposed in early 1900s by Paul Ehrlich, but it has not been until the development of monoclonal antibody technology, which started with the Milstein and Kohler [2] invention of hybridoma technology, that the creation of highly specific and potent cytotoxic agents has been possible.

ADCs combine the exquisite specificity of mAbs with highly potent chemotherapeutic compounds. An ADC is composed of a specific antibody to a target overexpressed in cancer cells, a potent cytotoxic molecule (payload), and a linker to covalently join the payload, in an inactive form, to the antibody; this allows cytotoxin activation when the antibody reaches the target cell generally by linker cleavage or antibody degradation in the lysosomal compartment. The combination of target, conjugation chemistry, linker, payload, and drug to antibody ratio (DAR) determines the efficacy and toxicity of the ADC (therapeutic index), and therefore developing a new ADC is a combinatorial problem that requires very fine-tuned and significant empirical optimization.

While there is widespread research to discover new drugs to use as payloads in ADCs, only tubulin inhibitors and DNA-interacting agents are now in clinical development. Tubulin inhibitors stop cell division by inhibiting tubulin polymerization. The two major types of tubulin inhibitors used currently for ADCs are auristatins (monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF)) and maytansine derivatives (DM1, DM2, DM3). *In vitro* potencies of auristatins are nanomolar to high subnanomolar [210] while maytansines show picomolar *in vitro* activities [211].

DNA-interacting payloads are the other major class of drugs used in ADCs. Calicheamicin is the only example of this class that has received FDA (Food and Drug Administration) approval, as payload for gemtuzumab ozogamicin (Mylotarg®). ADCs containing duocarmycins and pyrrolobenzodiazepines (PBDs) are also in development. As in the case of potent tubulin inhibitors, these DNA-interacting drugs are too toxic and their therapeutic window is too narrow to be used as stand-alone agents, but their high potency makes them attractive to be combined with the specificity of an antibody [212].

As essential as the antibody and the payload are, the conjugation technique, and the chemistry of the linker joining the drug with the antibody. The linker needs to be stable in blood and keep the payload inactive until the ADC reaches target cells; at the same time, the linker needs to deliver the payload, in active form, to the intracellular space of the target cell. Conceptually, there are two kinds of linkers, cleavable and noncleavable (for a review, see Ref. [213]). Cleavable linkers can be grouped into two categories, chemically labile linkers and enzymatically cleavable linkers. Chemically labile linkers can be acid-cleavable hydrazones designed to hydrolyze in the internal compartment of the target cell after internalization, but must be stable in neutral pH. Acid-cleavable linkers were used in gemtuzumab ozogamicin and inotuzumab ozogamicin. Another kind of chemically labile linkers use disulfide bonds that are more labile in the high intracellular glutathione concentration of cancer cells; ImmunoGen, Inc. uses this kind of linker in their DM1 or DM4 ADCs [214]. Enzymatically cleavable linkers join the payload to the antibody through a peptide that can be cleaved by lysosomal proteases and expected to be stable in plasma. Enzyme-cleavable linkers can use a self-emulating spacer between the linker and the drug. This spacer self-cleaves after protease action liberating the payload molecule in unmodified form. When used with an uncharged molecule like MMAE, the free payload can diffuse across the cell membrane and kill the nearby cell. This bystander effect can be beneficial when target expression is not homogeneous in the tumor or when killing the noncancerous matrix cell can increase efficacy. Noncleavable linkers rely on the complete degradation of the antibody in lysosomes to liberate the toxin with the amino acid fragment of the antibody and linker attached. Noncleavable linkers need to use toxins that remain active when attached to the linker–antibody amino acid, like DM1 and MMAF. These amino acid–linker–toxin complexes cannot diffuse from cell to cell and do not have bystander effect. The selection of the linker will influence the therapeutic index of the ADC, and it should take into consideration the drug, target, antibody, and tumor type. In general, noncleavable

linker ADCs will not have bystander effect, will be more stable in circulation, and will be less potent and less toxic for the organism than cleavable linker ADCs using the same antibody and equivalent payload.

Initially, the conjugation of the linker–payload used amino groups of natural antibody lysines [215] or sulfhydryl groups of antibody interchange cysteines [216]. Because of the large number of lysines in an antibody, this type of conjugation yields the most heterogeneous product, an average of four drugs distributed among the large number of accessible lysines in the antibody structure [217]. Conjugation to interchange cysteine is less heterogeneous, generally targeting an average of four drugs distributed in eight cysteines [216]. In both cases, the final preparation contains unconjugated antibodies that act as competitors and highly loaded molecules (six or more drugs per antibody) proven to display reduced PK and high nonspecific toxicity.

To increase the homogeneity of the final product, and therefore improve the pharmacological properties and allow for a better understanding of efficacy, toxicity, and PK/PD of the ADC, several site-specific methods are in development. All these methods promise to allow a precise DAR at well-defined positions [218–220].

The first ADC tested in the clinic was BR96-Dox [221], an anti-Lewis Y antigen humanized antibody conjugated to the DNA intercalation agent doxorubicin with a DAR of 8. This ADC failed to show sufficient clinical benefit [222], presumably because of the low potency of the drug ($IC_{50} = 0.1–0.2\,\mu M$ for human cancer cell lines [223]). New payloads for ADCs are selected to be much more potent, with IC_{50} in the low nanomolar to picomolar range. The first ADC that obtained regulatory approval was gemtuzumab ozogamicin (Mylotarg®, in 2000 [224]), which is a humanized anti-CD33 IgG4/k linked to a very potent enediyne antibiotic that binds to DNA minor grooves and produces double-strand breaks that activate cellular apoptosis. Gemtuzumab ozogamicin was voluntarily withdrawn from the US market because of a narrow therapeutic index and failure to demonstrate clinical benefit [225]. Not until 2011 did another ADC, brentuximab vedotin (Adcetris®), receive FDA approval, a chimeric mouse/human IgG1 anti-CD30 antibody conjugated to a highly toxic monomethyl auristatin E (tubulin inhibitor) using a cleavable linker and a DAR of 4 that showed remarkable clinical results in Hodgkin's lymphoma and anaplastic large cell lymphoma. Trastuzumab emtansine (Kadcyla®), a Herceptin-DM1 ADC, was approved by FDA in 2013. At this time, more than 30 ADC programs are known to be progressing through preclinical and clinical studies [226].

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