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Improving Antibody Therapeutics by Manipulating the Fc Domain: Immunological and Structural Considerations

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Abstract

Interactions between the crystallizable fragment (Fc) domain of antibodies and a plethora of cellular Fc receptors (FcRs) or soluble proteins form a critical link between humoral and innate immunity. In particular, the immunoglobulin G Fc domain is critical for the clearance of target cells by processes that include (*a*) cytotoxicity, phagocytosis, or complement lysis; (*b*) modulation of inflammation; (*c*) antigen presentation; (*d*) antibody-mediated receptor clustering; and (*e*) cytokine release. More than 30 Fc-engineered antibodies aimed primarily at tailoring these effects for optimal therapeutic outcomes are in clinical evaluation or have already been approved. Nonetheless, our understanding of how FcR engagement impacts various immune cell phenotypes is still largely incomplete. Recent insights into FcR biology coupled with advances in Fc:FcR structural analysis, Fc engineering, and mouse models that recapitulate human biology are helping to fill in existing knowledge gaps. These advances will provide a blueprint on how to fine-tune the Fc domain to achieve optimal therapeutic efficacy.

Contents

1. INTRODUCTION

Adaptive immunity plays an indispensable role in providing protection from infectious agents and in regulating immune responses. As of 2020, more than 100 antibody therapeutics and cell therapies have been approved by the US Food and Drug Administration or the European Medicines Agency, including 11 new approvals in the past year alone (1). Antibody-based drugs enjoy the advantages of highly specific target recognition through their antigen-binding fragment (Fab) domains, long persistence in circulation, and the ability to interact with various cell types of the immune system to induce highly potent effector functions. The latter two features of antibodies are driven by the binding of the crystallizable fragment (Fc) to a wide array of Fc receptors (FcRs) on immune and somatic cells and are the focus of this review.

Antibodies interact with a variety of FcRs, which in turn orchestrate a myriad of immune functions. In healthy humans, approximately 70% of circulating immunoglobulin (Ig) is of the IgG isotype, 20% is IgA, and the remaining 10% is IgM, IgD, and IgE. IgG and IgA are further classified into IgG1–4 and IgA1–2 subclasses, respectively. Each one of these isotype classes has a corresponding family of receptors. These receptors include FcγRs for IgG, FcαRI for IgA, FcμR for IgM, Fcμ/αRI for both IgA and IgM, and FcεRI for IgE. Because antibodies are glycosylated, they can also interact with lectin receptors, such as CD209 (DC-SIGN), CD23 (FcεRII), and dectin I, which have important immunological functions in certain settings. In addition, the major histocompatibility (MHC) class I–type neonatal FcR (FcRn) modulates the half-life and biodistribution of IgG, whereas the polymeric immunoglobulin receptor is required for transcytosis of IgA and IgM to mucosal surfaces. Moreover, TRIM21 is a cytosolic receptor for IgG involved in antiviral immunity. Additionally, there are three FcR-like receptors: FcRL3 and FcRL4 (which bind IgA) and FcRL5 (which binds IgG); however, their functional role is not fully understood. Finally, the complement protein C1q binds to the Fc domain of IgG and IgM and initiates the classical

complement cascade, thus bridging antibody-driven to complement-driven effector functions. This review focuses on the FcRs of IgG, and to a lesser extent on those of IgA, since they are of key relevance to protein therapeutics (2, 3). Currently, all approved antibody therapeutics are of the IgG isotype, although IgM- and IgE-based therapies have recently entered clinical development.

Except for FcγRI (discussed in Section 3.2), most FcRs have evolved to have low affinity toward their ligands and are only activated when interacting with multivalent immune complexes (ICs) consisting of several antibodies binding to a target that has multiple binding sites. The cellular response to ICs largely depends on the cell type and the array of FcRs it expresses. In turn, FcR expression is affected by the cytokine environment and the tissue niche in which a particular cell is found. In broad terms, antibody effector functions refer to the following immunological mechanisms (**Figure 1**):

1. The release of cytotoxic molecules aimed at killing the targeted pathogen is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) (FcγR-mediated) or complement-dependent cell-mediated cytotoxicity (CDCC) (complement-mediated). In natural killer (NK) cells, the cytotoxic molecules are primarily granzyme and perforin, whereas in neutrophils killing is triggered by the release of reactive oxygen species (ROS) and proteases, as well as by chromatin expulsion from the nucleus to form neutrophil extracellular traps (NETs) (4).
2. Target engulfment and endolysosomal destruction include antibody-dependent cell-mediated phagocytosis (ADCP) and complement-dependent cell-mediated phagocytosis (CDCP). In addition to antibody-opsonized cells or particles, small soluble ICs are eliminated via FcRs by endocytic processes, in a manner that does not appear to require cytoskeletal rearrangement, in contrast to phagocytosis (5). Antibody-dependent trogocytosis is a special case of target engulfment, whereby effector cells, most notably neutrophils, ingest a portion of the membrane and cytoplasmic material from the target cell. Multiple trogocytosis events eventually lead to a form of apoptotic death termed trogoptosis (6, 7).
3. Complement-dependent cytotoxicity (CDC) is mediated by the formation of the membrane attack complex (MAC) on the surface of target cells of viruses, leading to their lysis.
4. The release of cytokines, chemokines, or other immunomodulatory agents [e.g., tumor necrosis factor alpha (TNFα) or interferon gamma (IFNγ)] regulates the inflammatory milieu. In turn, this process is largely dependent on the local inflammatory environment, the coactivation of other innate receptors, and the effector cell type (8).
5. Agonistic Fc-mediated clustering of cell surface receptors can trigger either activating or apoptotic signals (9).
6. Adaptive immune responses are modulated through antigen presentation, B cell activation, and induction of tolerance (10). Also, mounting evidence indicates that ICs have direct effects on T cells, which until recently had been thought not to express FcγRs (11, 12).

During evolution, FcRs appeared shortly after the emergence of Ig, and the expansion of antibody isotypes resulted in diversification of the FcR repertoire (13). While the first Ig genes are found in cartilaginous fish, various FcR homolog genes are found in bony fish, which evolved soon after. The FcγR family of receptors for IgG antibodies is found only among mammals. There is evidence of ongoing evolutionary pressure on the FcγR gene family, as humans carry the *FCGRIIC* [functional in only 15–18% of the Caucasian population (14)] and *FCGRIIIB* genes, which are not found in other primates. There is substantial genetic heterogeneity throughout the low-affinity *FCGR* locus among the human population, resulting from single-nucleotide polymorphisms (SNPs) and gene copy-number variations. Extensive evidence suggests that *FCGR* haplotypes are strongly correlated with increased susceptibility to certain autoimmune

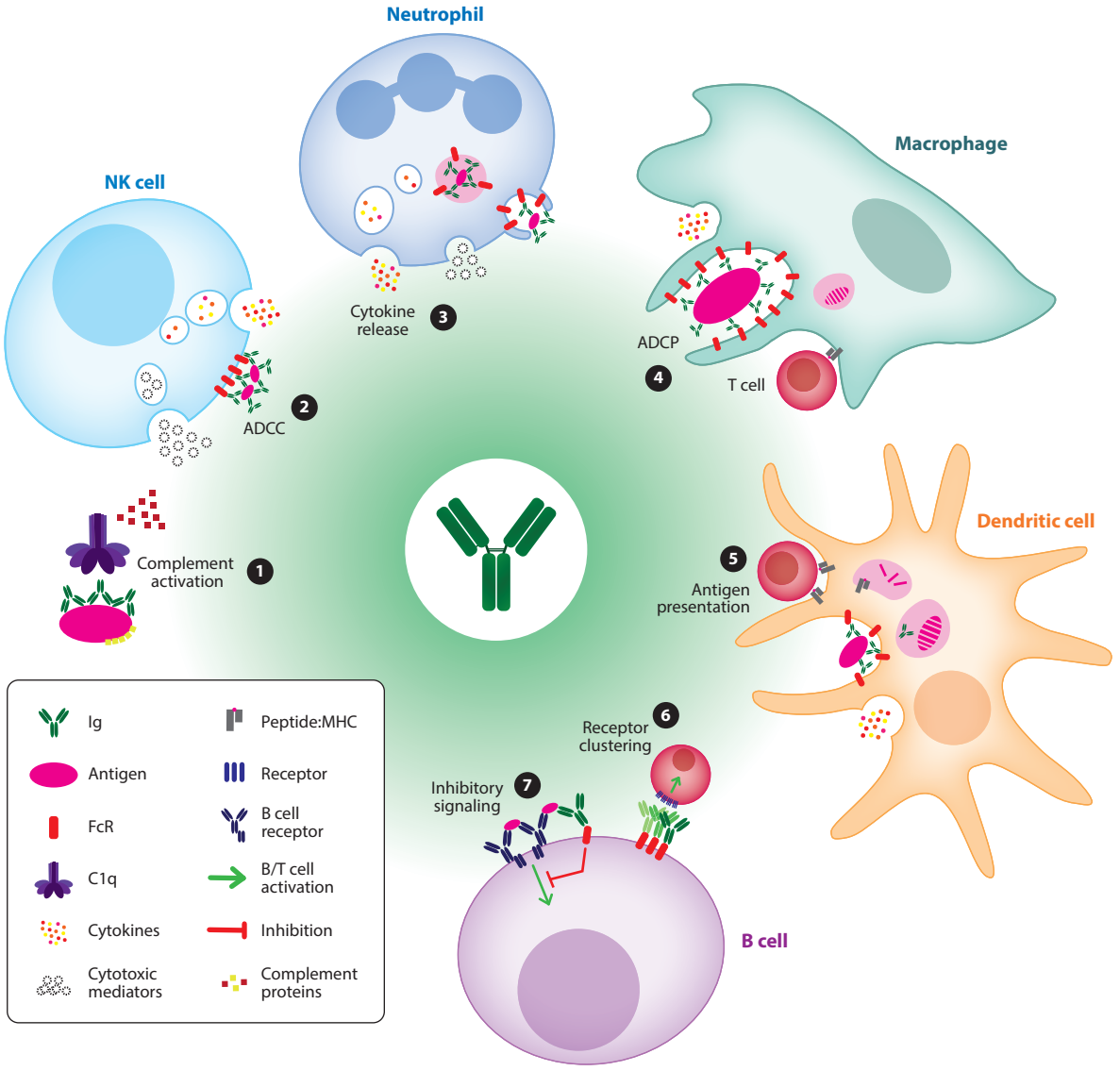

Figure 1

Ig:FcR engagement triggers a spectrum of effector functions, bridging innate and adaptive immune responses. Ig binding on the antigen initiates the complement cascade (❶), activating NK cells to release cytokines and cytotoxic proteins and leading to ADCC (❷). Neutrophils engulf and degrade ICs while releasing inflammatory mediators (❸). Ig-coated pathogens are phagocytosed by macrophages (❹) and dendritic cells (❺). This facilitates the presentation of antigen by MHC class molecules, leading to T cell stimulation. FcRs can cause agonistic antibody-driven receptor clustering (e.g., 4-1BB on T cells) (❻), leading to receptor activation. Finally, inhibitory FcR signaling (❼) can counteract B cell receptor activation, thus promoting tolerance. Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; FcR, Fc receptor; IC, immune complex; Ig, immunoglobulin, MHC, major histocompatibility; NK, natural killer.

disorders, protection from infectious agents, and response to tumor immunotherapy (15, 16), further underscoring the critical role of FcRs in potentiating antibody-mediated functions.

Modifications in the Fc that impact FcR engagement have been shown to drastically affect the efficacy of antibody therapeutics. Efforts to alter either the composition of the glycan or

Table 1 Select examples of therapeutic antibodies having Fc domains engineered to modulate effector FcR functions

Main function	Antibody	Company	Target	Isotype and Fc modifications	Lead indication	Trial phase/approval
Cytotoxicity	Obinutuzumab (Gazyva®)	Genentech Inc.	CD20	Humanized IgG1, afucosylated (ADCC)	CLL	Nov. 2013
	Tafasitamab (Monjuvi®)	MorphoSys Inc.	CD19	IgG1/2 chimera, S239D/I332E (ADCC)	DLBCL	July 2020
	Margetuximab (Margenza™)	MacroGenics	HER2	Chimeric IgG1, L235V/F243L/R292P/Y300L/P396L (ADCC)	HER2+ BC	Dec. 2020
	Elipovimab	Gilead	HIV gp120	IgG1, G236A/S239D/A330L/I332E+M428L/N434S (ADCC, ADCP, half-life extension)	AIDS	Phase I
Agonistic	HexaBody®-DR5/DR5	Genmab	DR5	Mix of two mAbs, E430G (clustering, CDC)	Solid tumors	Phase I
Antagonistic	Atezolizumab (Tecentriq®)	Genentech Inc.	PD-L1	IgG1, aglycosylated N297A (silenced)	UC	May 2016
Clearance	Ravulizumab (Ultomiris®)	Alexion Pharmaceuticals	C5	IgG2/4 chimera, M428L/N434S (half-life extension)	PNH, aHUS	Dec. 2018
Clearance/immunomodulatory	Xmab7195	Xencor/Aimmune Therapeutics	IgE	Humanized IgG1, S267E/L328F (clearance, FcγRIIb agonism)	AA	Phase I
Immunomodulatory	Efgartigimod	Argenx	NA	IgG1 Fc, M252Y/S254T/T256E/H433K/N434F (FcRn agonism)	MG, ITP	Dec. 2021
	M230/CSL730	CSL Behring	NA	Trivalent IgG1 Fc (FcR blocking)	Autoimmunity	Phase I
	M254	Momenta Pharmaceuticals/Janssen	NA	Polyclonal IgG, hypersialylated (anti-inflammatory)	ITP	Phase I/II

Abbreviations: AA, allergic asthma; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; aHUS, atypical hemolytic uremic syndrome; AIDS, acquired immunodeficiency syndrome; BC, breast cancer; CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; Fc, crystallizable fragment; FcR, Fc receptor; HER2, human epidermal growth factor receptor 2; Ig, immunoglobulin; ITP, immune thrombocytopenic purpura; mAb, monoclonal antibody; MG, myasthenia gravis; NA, not applicable; PNH, paroxysmal nocturnal hemoglobinuria; UC, urothelial carcinoma.

the protein backbone of the Fc have been employed to create second-generation therapeutics, with properties such as increased efficacy, lower toxicity, extended half-life for easier administration, and lower manufacturing costs (3) (**Table 1**). The glycoengineered anti-CD20 antibody obinutuzumab was the first therapeutic with a modified Fc domain to be approved for the treatment of chronic lymphocytic leukemia in 2013, approximately 16 years after the approval of the first CD20-depleting monoclonal antibody, rituximab. More recently, the Fc-engineered antibody margetuximab [anti–human epidermal growth factor receptor 2 (HER2)] was approved for the treatment of metastatic HER2-positive breast cancer patients who had been unsuccessfully treated before with another anti-HER2 agent. Both antibodies have shown more effective killing of target cells relative to antibodies that target the same epitope but do not have engineered Fc domains. Examples of antibodies that are Fc engineered for improved effector functions are shown in **Table 1**.

2. THE CLASSICAL Fc RECEPTORS: EXPRESSION, BINDING, AND FUNCTION

2.1. The Effector FcγR Family

In humans, the FcγR family consists of six highly homologous receptors that bind to IgG antibodies. The biochemistry of the effector FcγRs has been reviewed extensively (17, 18). Briefly, FcγRI (CD64), FcγRIIa and FcγRIIc (CD32A/C), and FcγRIIIa (CD16A) are activating FcγRs in humans, primarily inducing proinflammatory responses. FcγRI and FcγRIIIa associate with an accessory signaling coreceptor (FcRγ/FcεRγ common chain on myeloid or CD3ζ /CD247 on NK cells) that contains an immunoreceptor tyrosine-based activation motif (ITAM), which recruits tyrosine kinases to initiate a signaling cascade, as discussed below (Section 3.1). FcγRI is the only receptor with three extracellular Ig domains, whereas all other FcγRs have two (17). Binding to the IgG Fc occurs at the relatively conserved interface between the first and second extracellular Ig domains of the FcγRs. In contrast to FcγRI and FcγRIIIa, the activating receptors FcγRIIa/c have an ITAM within their cytoplasmic domain (19). FcγRIIb (CD32B) is the sole inhibitory FcγR that contains a canonical immunoreceptor tyrosine-based inhibitory motif (ITIM) (**Figure 2**). FcγRIIIb is a glycosylphosphatidylinositol-anchored protein that is devoid of a signaling domain and that is expressed solely on polymorphonuclear cells. All FcγRs have medium to low affinity toward IgG (K_D of 0.5–5 μM), except for the high-affinity FcγRI (K_D ~3 nM) (20). However, it should be emphasized that these affinity values are based on biophysical measurements using recombinant receptors expressed in widely used cell lines, such as HEK293 cells, and therefore they should be interpreted with caution as it is now becoming apparent that in primary cells, posttranslational modifications can significantly affect antibody affinity to FcRs (18). As an example, the presence of Man5 *N*-glycans on FcγRIIIa results in much higher affinity to IgG1, and in the case of afucosylated IgG1, the binding affinity is very high, with a K_D similar to that of the high-affinity receptor FcγRI (21). Given the differences in FcγR glycosylation patterns among different cell types (22, 23), knowledge of the glycan composition of both the FcR and the antibody is important for understanding effector functions.

Common SNPs in FcγRs can affect antibody binding strength, and the resulting differences in affinity translate into altered therapeutic efficacy of antibody drugs. In the case of FcγRIIIa, a Val at position 158 (FcγRIIIa$_{V158}$) results in two- to threefold higher affinity for IgG1 relative to F158 (FcγRIIIa$_{F158}$) (20). On average, 36% of all humans carry the high-affinity allele, with different distributions among various ethnic groups. Breast cancer patients homozygous for FcγRIIIa$_{V158}$ show better response rates when treated with trastuzumab (24). Similar effects have been reported after rituximab administration in patients with non-Hodgkin's lymphoma (25). The most common polymorphism in FcγRIIa is at position 131 (His or Arg). The FcγRIIa$_{H131}$ allelic variant occurs at a frequency of 44–67% in the human population and results in slightly higher affinity for IgG1 (20). Homozygosity for the high-affinity allele is associated with improved response to IgG1 antibody therapeutics (24) but is also associated with increased susceptibility to certain autoimmune diseases, including Kawasaki disease, Guillain–Barré syndrome, ulcerative colitis, and childhood immune thrombocytopenic purpura (ITP). Interestingly, the low-affinity allele FcγRIIa$_{R131}$ has also been linked to autoimmunity, such as systemic lupus erythematosus (SLE), ITP, and IgA nephropathy, possibly resulting from reduced capacity for FcγRIIa-dependent clearance of circulating ICs (15, 16).

FcγRs display different binding affinity and selectivity toward the various IgG subclasses (20, 26). Monomeric IgG1 and IgG3 can engage all FcγRs, with preferential binding toward FcγRIIa and FcγRIIIa over FcγRIIb and FcγRIIIb. Conversely, IgG2 can bind to the FcγRIIa$_{H131}$ allele but shows lower binding toward FcγRIIa$_{R131}$, FcγRIIb, and FcγRIIIa and no binding to FcγRI

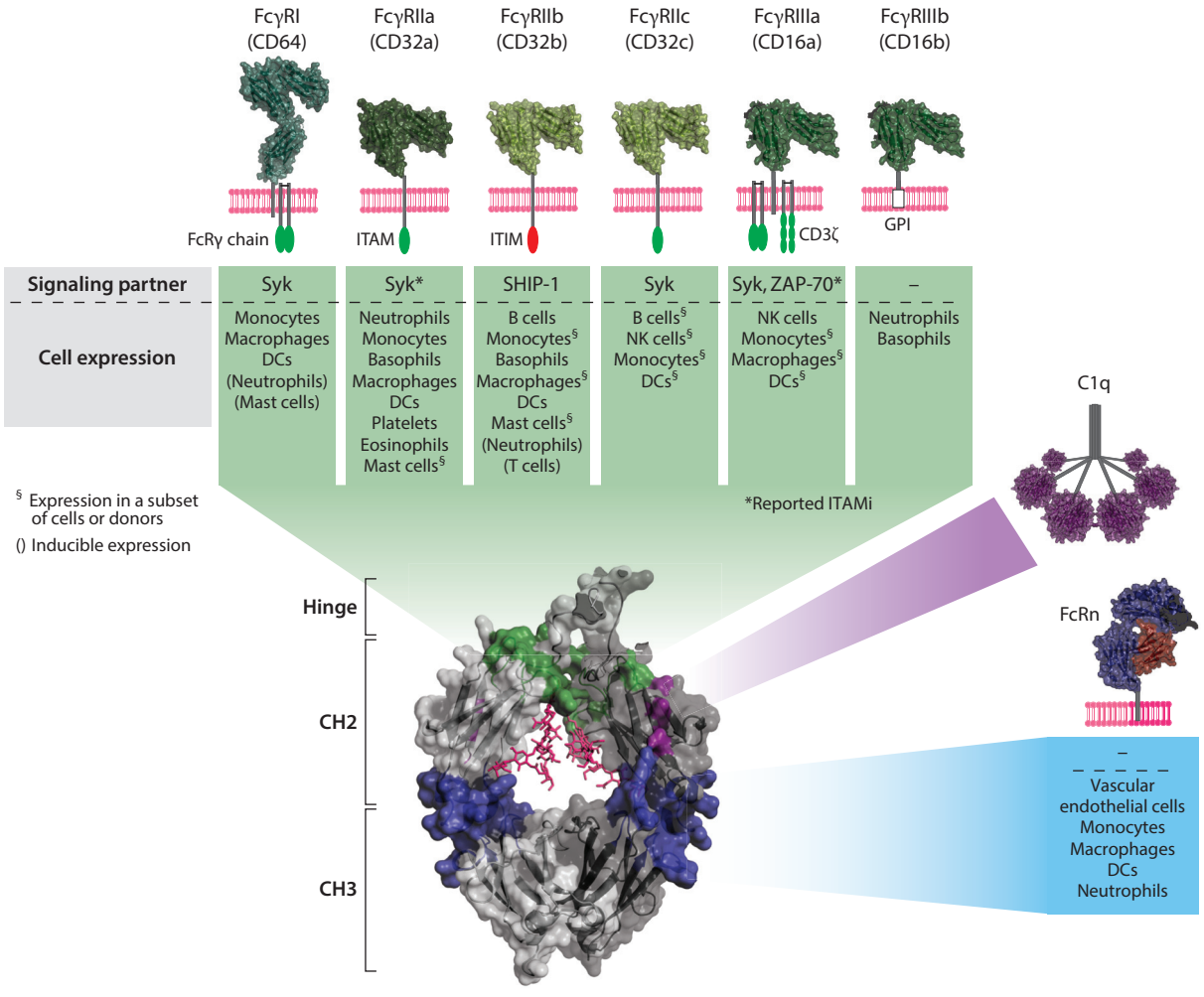

Figure 2

Structure of the IgG1 Fc (PDB ID: 1HZH) and the Fc binding receptors. The two chains of the Fc are colored light and dark gray, and the N297 attached glycan is shown in pink. The binding sites of the FcγRs (*green*), C1q (*purple*), and FcRn (*blue*) on the Fc are highlighted. Structures of the extracellular domains of the FcγRs (FcγRI PDB ID: 3RJD, FcγRIIa PDB ID: 3R5Y, FcγRIIb/c PDB ID: 2FCB, FcγRIIIa/b PDB ID: 5VU0), FcRn (PDB ID: 5WHK, α-chain in *blue*, β2m in *red*), and C1q head (PDB ID: 1PK6) are also depicted. The cellular expression and signaling molecules directly downstream of the Fc receptors are indicated. FcγRIIIa can signal through CD3ζ /ZAP-70 in NK cells, as well as the FcRγ common chain. There is evidence of inhibitory ITAMi signaling for FcγRIIa and FcγRIIIa. Abbreviations: DC, dendritic cell; Fc, crystallizable fragment; FcγR, Fc gamma receptor; FcRn, neonatal Fc receptor; GPI, glycosylphosphatidylinositol; IgG1, immunoglobulin G1; ITAM, immunoreceptor tyrosine-based activation motif; ITAMi, inhibitory ITAM signaling; ITIM, immunoreceptor tyrosine-based inhibition motif; NK, natural killer; PDB ID, Protein Data Bank identifier; SHIP-1, SH2 domain-containing inositol polyphosphate 5-phosphatase 1; Syk, spleen tyrosine kinase; ZAP-70: zeta-chain-associated protein kinase 70.

or FcγRIIIb. Finally, IgG4 can engage FcγRI but has minimal binding toward the low-affinity FcγRs. It is important to note than even though IgG2 and IgG4 have minimal binding to the monomeric, low-affinity FcγRs in solution, high-avidity ICs formed by antibodies of these isotypes readily bind to cells expressing moderate to high levels of these receptors (20, 27). Still, as a

consequence of their weak FcγR-binding profile, IgG2 and IgG4 are less inflammatory, which may be the reason why class switching to these isotypes occurs after IgG1 or IgG3 following infection or vaccination (28). As discussed below, antibody drugs that primarily function by blocking receptor:ligand interactions are almost exclusively of the IgG2 or IgG4 subclass or engineered IgG1 antibodies with mutations that preclude FcR or C1q binding to avoid cytotoxicity (see Section 5.1) (29).

As in the FcγR genes, there is substantial genetic variability in the antibody heavy chain genes, with at least 27 relatively common IgG allotypes in humans. Some of these allotypes have been shown to slightly affect FcR affinity and therefore impact target cell clearance potency in vitro, especially in the case of IgG3 (30).

As with their affinity to IgG, the FcγR expression patterns on cells are tightly regulated to avoid extensive tissue damage by circulating IgG. The expression profile of FcγRs in circulating and tissue-resident hematopoietic cells is complex (**Figure 2**) (31). Briefly, lymphoid cells display a limited FcR expression profile, with mature B cells or plasma cells constitutively expressing only FcγRIIb, NK cells expressing FcγRIIIa, and only some subsets of T cells expressing generally low levels of activating receptors or FcγRIIb. In contrast, cells of the myeloid lineage show greater FcγR expression plasticity. FcγRIIa is expressed by most myeloid-lineage cells, including platelets, but not by red blood cells (RBCs). Cells expressing FcγRIIa typically also express low levels of FcγRI and the inhibitory FcγRIIb, with the expression of these receptors often upregulated under inflammatory conditions. FcγRIIIa is found on monocytes and macrophages, whereas FcγRIIIb is expressed only on neutrophils and basophils (32). Resting neutrophils do not express detectable levels of FcγRIIb and FcγRI, but these receptors can be induced during an inflammatory response (33). Apart from leukocytes, FcγRIIb is the sole receptor expressed in liver sinusoidal endothelial cells (LSECs), where it plays an integral role in removing circulating ICs from the bloodstream (34).

It is important to emphasize that for each cell type shown in **Figure 2**, there are numerous subsets that differ widely in terms of FcR expression and effector function potency. For example, the nonclassical monocytes ($CD16^+$, $CD14^{lo}$) are phenotypically different from their classical counterparts ($CD16^-$, $CD14^{high}$), which express higher levels of FcγRI (31, 35). Functionally, $CD16^+$ monocytes have been reported to mediate better tumor cell killing and phagocytosis (35, 36). Moreover, the murine counterparts of nonclassical monocytes ($Ly6C^{lo}$), which express mFcγRIV (a functional homolog of human FcγRIIIa), are responsible for a major part of cell-mediated IgG depletion of circulating target cells in vivo (37).

The diversity of expression of FcRs on macrophages is noteworthy and is related to their two different cellular lineages: self-renewing, tissue-resident macrophages and monocyte-derived macrophages. Tissue-resident macrophages are embryonically derived and have the ability to self-replicate, whereas monocyte-derived macrophages originate from the bone marrow, colonize tissues under inflammatory conditions, and cannot replicate but instead are continuously replenished from circulating monocytes (38). Human tissue-resident macrophages are difficult to extract; therefore, little is known about their FcR expression. Instead, macrophage functions have been studied primarily using monocytes differentiated into macrophages in vitro (35, 39–41). Caution is advised when interpreting results from monocyte-derived macrophages, as slight differences in the culturing and differentiation protocols can significantly affect FcR expression and the downstream effector responses (35, 42). The limited data show that there are significant differences in the FcγRI and FcγRIIIa expression levels between tissue-resident and monocyte-derived macrophages in humans (41, 43). For example, there are at least two distinct macrophage subsets in the spleen, namely, $CD163^+$ red pulp macrophages and $CD169^+$ perifollicular zone macrophages (41). Red pulp macrophages show low FcγRI and FcγRIIb expression and high

FcγRIIa and FcγRIIIa expression, and they are very effective in the FcγR-dependent phagocytosis of IgG-opsonized erythrocytes (41).

2.2. The Neonatal FcR

FcRn belongs to the MHC class I–receptor family and is expressed primarily on vascular endothelial cells but also on epithelial cells, macrophages, and other cell types (44). Originally named for its role in IgG transport from the mother to the fetus or neonate, FcRn is responsible for the long half-life of IgG in circulation (21–27 days). It carries out this responsibility by interacting with IgG only at endosomal pH (6.0) and not at physiological pH (7.4) (45). Following pinocytosis or receptor-mediated endocytosis, IgG in acidic endosomes binds FcRn and is recycled back to the plasma membrane, where it is released at the physiological pH of the extracellular space. This intricate process protects IgG from lysosomal degradation. In addition, FcRn is responsible for transporting IgG across epithelial barriers, allowing for IgG delivery on mucosal surfaces and across the placenta (46). Finally, due to its endosomal shuttling ability, FcRn plays an important role in antigen cross-presentation and as a co-receptor of the activating effector FcRs (46, 47).

2.3. FcαRI

IgA interacts with effector cells primarily through FcαRI (CD89), which is expressed in most mammals including rats but surprisingly not in mice (13). Although FcαRI has two extracellular Ig domains and signals by associating with the FcRγ common gamma chain, as is the case for FcγRI and FcγRIIIa, it shares only ~20% sequence homology with members of the FcγR family (48). Monomeric IgA1, IgA2, and dimeric IgA1/2 can bind FcαRI with medium to low affinity; however, the secreted IgA that is present in mucosal surfaces completely loses binding to FcαRI (49). No FcαRI SNPs that affect IgA binding have been reported; however, the mutation S248G in the cytoplasmic domain has been associated with increased susceptibility to SLE (50). FcαRI is expressed in most subsets of myeloid cells, primarily in circulating neutrophils and monocytes, but displays lower expression in tissue-resident macrophages and dendritic cells (DCs) and as such is not very proficient in mediating FcαRI-driven antigen presentation (51). FcαRI is capable of stimulating stronger neutrophil cytotoxicity of IgA-opsonized targets, compared with IgG-driven cytotoxicity (52).

2.4. C1q

Though not a cell receptor, C1q is a functionally important Fc-binding protein for therapeutic purposes, as its interaction with IgG ICs (or IgM) can initiate the classical complement cascade (53). C1q primarily binds to IgG1 and IgG3, but not IgG2 or IgG4. However, when IgG2 antibodies form high-density ICs they can mediate complement-dependent protection from bacterial infection in vivo, perhaps via the activation of the lectin-binding pathway (54). The hexameric C1q molecule requires binding of at least four Fc molecules to initiate the classical complement cascade. The formation of C1q:antibody complexes is strongly dependent on geometric constraints on the antigen surface. Once activated, the antibody:C1 complex catalyzes the formation of the C3 convertase, which leads to C3b deposition on the target surface and ultimately results in the release of anaphylatoxins (e.g., C5a) and also the formation of the MAC. Surface-deposited complement fragments can be recognized by complement receptors on myeloid cells and lymphocytes, which in turn give rise to complement-dependent effector functions (cytotoxicity and/or phagocytosis). While the significance of CDCC for the elimination of antibody-opsonized tumor cell killing has

been underappreciated, recent studies have suggested that its importance can be comparable to that of FcγR-driven ADCC, in terms of magnitude and kinetics (55).

3. SIGNALING IDIOSYNCRASIES OF FcRs

3.1. Overview

Signaling of FcRs, whether activating or inhibitory, is analogous to B cell receptor (BCR) and T cell receptor activation. Briefly, upon engaging immune complexes, activating FcγRs (or FcαRIs) cluster together and their ITAMs are phosphorylated by Src-family kinases. Phosphorylated ITAMs provide a docking site for the SH2-containing kinases Syk and ZAP-70 (found in lymphocytes), which in turn can phosphorylate multiple downstream kinases and adaptor molecules, such as phospholipase C gamma (PLCγ), Bruton's tyrosine kinase (BTK), Vav, and phosphoinositide 3-kinase (PI3K). PI3K activity converts phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which acts as a docking site for PLCs, BTK, Akt, and other kinases or phosphatases containing a PIP3-binding pleckstrin homology domain. Activated PLCγ catalyzes the conversion of the membrane-bound PIP2 to the second-messenger molecules diacylglycerol and inositol triphosphate (IP3), which results in rapid Ca^{2+} flux and activation of NF-κB, NFAT, or AP1 transcription factors (56). In phagocytic cells, ITAM signaling leads to actin polymerization and induction of phagocytosis (57, 58). The actin network that is formed pushes the plasma membrane around the target, forcing more FcγRs on the emerging membrane protrusion to participate in further cytoskeletal reorganization, resulting in complete engulfment of the antigen. The phagocytotic cup finally closes by a PI3K-dependent membrane fusion process and thus forms the phagosome (59).

Fine-tuned regulation of the potent activating signals stemming from FcR engagement is crucial to avoid unwanted tissue damage. One mechanism for controlling the activation of IC-sensitized effector cells is via the engagement of FcγRIIb, the sole inhibitory FcγR, which functions as a negative signaling regulator for activating FcRs in myeloid cells and the BCR in B cells (17). As with activating FcγRs and upon binding to ICs, the cytoplasmic ITIM of FcγRIIb is phosphorylated by Src kinases, which can then recruit SHIP-1/SHIP-2. Unlike most ITIM-bearing receptors that recruit the tyrosine phosphatases SHP-1 and SHP-2, the phosphorylated cytoplasmic tail of FcγRIIb can only accommodate SHIP-1/2 binding (60). SHIP-1 and SHIP-2 are inositol 5-phosphatases that convert PIP3 back to PIP2, counteracting PI3K's function and effectively blocking all downstream signaling. FcγRIIb can also function independently of the ITIM phosphorylation and the recruitment of phosphatases in B cells, by physically colocalizing with the BCR in lipid rafts and thus preventing CD19 from providing its costimulatory signals (61).

An important and often overlooked consideration is that ITAM-bearing FcRs can also initiate inhibitory ITAM signaling (ITAMi) (56). This signaling process occurs under conditions that lead to low levels of FcγR clustering, resulting in incomplete phosphorylation of ITAMs, which cannot recruit Syk and instead bind to the phosphatase SHP-1 (62). SHP-1 is a tyrosine phosphatase that directly dephosphorylates various proximal substrates, such as Igα/β, Syk, FcR ITAMs, and PI3K, thus preventing downstream signaling. This inhibitory role of ITAM-bearing receptors has been reported for the FcRγ common chain signaling receptors FcαRI (63) and FcγRIIIa (64) and also for FcγRIIa, which has its own intracellular ITAM domain (62). Although not completely understood, ITAMi helps maintain immune homeostasis and protect against autoimmune or inflammatory diseases by increasing the signaling threshold required for cell activation (65).

ITAMi is a perfect example of how sensitive FcR signaling is to extrinsic factors. In addition to IC valency, the micro-organization of the forming immune synapse—which is determined by factors such as the lipid composition (66), the density of receptors, and the presence of

other signaling mediators (67)—is crucial in determining the strength and outcome of the FcR signaling. For example, Bakalar et al. (68) elegantly showed that physical exclusion of the bulky CD45 phosphatase from the immune synapse is a prerequisite for strong FcγRIIa-mediated phagocytosis, thus linking FcγR signaling with the height of the targeted epitope. Finally, FcR signaling can be intimately controlled by external cytokine and Toll-like receptor (TLR) ligands [in a process known as inside-out signaling, which has been reviewed elsewhere (69, 70)].

3.2. Dissecting FcR Signaling and Function on Myeloid Cells

While the sequence of signaling events following FcR ligation by ICs is reasonably well understood, the precise cellular phenotypes triggered by each effector receptor are determined by additional mechanisms that have not been fully elucidated (71). As most innate immune cells express multiple FcRs, the binding of ICs results in co-clustering of different FcRs, and thus the ensuing cellular response effectively represents the integrated effect produced by multiple convergent or competing signaling pathways. Understanding the events that are triggered by the engagement of each FcR is very relevant for the engineering of protein therapeutics since tailoring the binding of the Fc domain to the desired FcRs is critical to achieve a particular outcome. In this section, we highlight some unique aspects of individual FcR signaling and examine how they may be influencing effector phenotypes.

It is important to note that the distinct functional role of each FcγR has been inferred primarily from studies using antibody fragments that recognize a specific FcγR via their Fab. Use of these antibody fragments (in combination with secondary anti-Ig antibodies for cross-linking) mimics IC binding by directly engaging and activating one receptor. Alternatively, they are used in the presence of IgG ICs to block a subset of FcγRs, thus indirectly achieving selective FcγR engagement. Such blocking is necessitated, since IgG molecules can engage with all FcγRs at the same time. For example, blocking $F(ab')_2$ antibodies to FcγRIIb are routinely used to separate the effect of engaging the activating receptors from the inhibitory receptor (39). However, the use of blocking $F(ab')_2$ fragments to one or more FcγRs can alter the distribution of surface proteins on the plasma membrane, and in certain cases this may even result in inadvertent Fab-mediated activation (72). Similarly, agonistic, anti-FcγR monoclonals activate FcRs with different affinity and geometry compared with IgG ICs, thus likely affecting the resulting signal transduction events.

One perplexing aspect of FcγR biology is the function of the high-affinity FcγRI. The dissociation constant of FcγRI for IgG1 is approximately 1×10^{-9} M, which is several orders of magnitude below the concentration of IgG in serum ($0.5–1 \times 10^{-4}$ M), suggesting that FcγRI on circulating cells is normally occupied by monomeric IgG. This is likely the case for cells within tissues as well (73). FcγRI bound to monomeric IgG is rapidly internalized and recycled to the cell surface, without the need for ITAM signaling (74). This perhaps explains the low expression levels of FcγRI in circulating effector cells during homeostasis (except from classical monocytes) (31). Of note, tissue-resident alveolar, cervical, or ex vivo generated monocyte-derived macrophages express higher levels of FcγRI (41, 43, 75), unlike tissue-resident splenic or bone-marrow macrophages that are constantly exposed to IgG. To trigger ITAM signaling, FcγRI requires cross-linking with IgG ICs, the same as for the low-affinity FcγRs. FcγRI cross-linking can potentiate monocyte and macrophage phagocytosis (41, 76), neutrophil cytotoxicity (77), and anaphylaxis (78). Not surprisingly, the addition of low amounts of IgG-containing human serum in in vitro assays completely abrogates phagocytosis via FcγRI (41, 76). In addition, FcγRI is sensitive to inside-out signaling, as it has been shown that certain cytokines increase its affinity to IgG ICs by enhancing receptor clustering on the cell surface (79).

Another way to look at the functional role of FcγRI is to compare the effects of its selective engagement with those of the low-affinity, activating receptor FcγRIIa, since both are co-expressed

on the same myeloid cells (**Figure 2**). Earlier evidence suggests that macrophage and monocyte phagocytosis of RBCs (41), platelets (80), and antigen-coated beads (76) is primarily mediated by FcγRI and not by FcγRIIa. This result is consistent with the poor phagocytosis of tumor cells by IgG2 antibodies, which can only engage $FcγRIIa_{H131}$ and not FcγRI (81). In contrast, smaller immune complexes are readily internalized only by FcγRIIa, but it remains unclear whether this effect requires ITAM signaling or actin reorganization (82). Unlike FcγRIIa, FcγRI resides in lipid rafts (83) and colocalizes with the inhibitory receptor signal-regulatory protein alpha (SIRPα) under resting conditions (84). As such, large ICs have to somehow overcome SIRPα:CD47 interactions that mediate the "do not eat me" effect in order for ADCP to occur (84). Apart from endocytosis or phagocytosis, FcγRIIa is solely responsible for the release of proinflammatory cytokines when macrophages, DCs, and monocytes are exposed to either large ICs (or plate-coated IgG) or the insoluble fraction of heat-aggregated IgG, in parallel with various TLRs or cytokine stimuli (85). Interestingly, FcγRI binding to similar ICs (plate-coated IgG) has been shown to dampen the response to IFNγ stimulation (86, 87). It is therefore evident that, even though both contain ITAM signaling domains, FcγRI and FcγRIIa transmit different signals to the FcR-bearing cell. Thus, the term "activating" FcγR needs further classification to fully capture the context-dependent role of each receptor.

Differences in the downstream signaling events following FcγRI or FcγRIIa stimulation have been reported. Selective cross-linking of FcγRI on IFNγ-treated U937 monocytic cells results in transient mobilization of Ca^{2+} from intracellular stores, whereas FcγRIIa stimulation shows longer calcium influx that is partially inhibited upon extracellular calcium depletion (88, 89). FcγRIIa signaling is mediated by PLCγ activity and concomitant intracellular IP3 increase, while FcγRI utilizes phospholipase D and the Ca^{2+}-independent isoforms of protein kinase C. Phospholipase D activity can also initiate the sphingosine kinase pathway and is involved in differential membrane trafficking (88, 89). Also, FcγRI-targeted ICs colocalize with the late-endosomal markers HLA-DR and LAMP-1, translating to more efficient antigen presentation to $CD4^+$ T cells, compared with ICs internalized by FcγRIIa (88). Interestingly, this unique signaling pattern of FcγRI on IFNγ-treated monocytes is lost upon differentiating monocytes toward macrophages (90) or DCs (91), thus highlighting the importance of cell type and costimulatory signals in regulating FcR functions.

FcγRIIa is crucial for initiating neutrophil inflammatory functions upon IC exposure. On the other hand, the role of FcγRIIIb remains contested (33). Resting neutrophils express ~20,000 copies of FcγRIIa, more than 200,000 copies of FcγRIIIb, and very low levels of FcγRI (31). Upon treatment with IFNγ or granulocyte colony-stimulating factor (G-CSF), FcγRI is upregulated while FcγRIIIb expression is decreased (92). Since FcγRIIIb does not have a signaling domain, this finding led to the notion that its main role is to dampen or reduce the signaling of other activating receptors during homeostasis, by acting as a decoy FcγR. Indeed, FcγRIIIb has been shown to decrease the cytotoxicity and trogocytosis of antibody-opsonized tumor cells co-cultured with neutrophils (93), in a process that likely involves recruitment of the phosphatase SHP-2 (94). FcγRIIIb is nonetheless capable of independently clearing soluble ICs by fluid-phase endocytosis (95). Interestingly, FcγRIIIb blocking reduces the phagocytosis of small IgG-coated particles or the IgG-driven generation of ROS and the formation of NETs by neutrophils (76, 95–97). Thus, in certain cases, FcγRIIIb might cooperate with FcγRIIa, but the exact conditions required for this are still incompletely understood.

It is easier to understand the signaling differences between FcαRI and FcγRI or FcγRIIa, as IgG antibodies do not bind to FcαRI and vice versa. When formatted with an IgA constant region, cytotoxic antibodies such as trastuzumab, cetuximab, and rituximab show enhanced ADCC by neutrophils, compared with their IgG counterparts (98). The lower activity of IgG relative to IgA

with neutrophils as effectors might be related to the co-engagement of FcγRIIIb, which may be serving as a decoy receptor for IgG. Alternatively, the different signaling motifs between FcαRI and FcγRIIa could result in lower activation of neutrophils by IgG. Like FcγRI signaling, FcαRI signaling is transduced by the FcRγ common chain and is regulated by inside-out signaling (99). This hypothesis is supported by the finding that in FcγRI-positive cells, macrophage phagocytosis and monocyte ADCC are equally strong with IgG and IgA antibodies (100), despite the lower expression of FcαRI relative to FcγRs on these cells (40, 98). This can be potentially explained by the ability of one IgA Fc to engage two FcαRI molecules, leading to stronger receptor cross-linking, compared with the 1:1 IgG:FcγR interaction.

4. THE MODULAR STRUCTURE OF THE Fc DOMAIN AND ITS ROLE IN FcR BINDING

Biochemical and structural studies have elucidated many of the key interactions responsible for the binding of FcγRs to the IgG1 Fc domain, and since they have been summarized in a number of excellent reviews (101, 102), only a few aspects of Fc:FcγR interactions of particular relevance are mentioned here.

Each chain of the Fc consists of an N-terminal flexible hinge, followed by two Ig domains. The two C-terminal CH3 domains form extensive hydrophobic contacts in the interface between the two chains; these contacts are key for the overall inverted horseshoe structure of the Fc domain (**Figure 2**). In IgG antibodies, the lower hinge region and the top of the two CH2 domains form the binding site of FcγRs and C1q. Surprisingly, and despite the sequence divergence among IgG isotypes, the contact sites of all IgG isotypes to the six members of the FcγR family are conserved, with the largest contribution to receptor binding affinity arising from the lower hinge (103). FcγR binding to IgG is asymmetric with respect to the two heavy chain polypeptides, A and B. Chain B interacts with FcγRs primarily by forming salt bridges and via hydrogen bonding with residues in the B/C and C′/E loops of CH2 in addition to van der Waals interactions with the lower hinge. Chain A forms a tight interaction between Pro329 in the F/G loop of the Fc and two conserved Trp residues (e.g., Trp87 and Trp110 in FcγRIIIa) found in all FcγRs, termed a proline sandwich (104). In addition, the lower hinge of chain A participates in hydrogen bonds and in hydrophobic interactions. The high affinity of FcγRI for IgG1 is attributed to the Leu235 residue of IgG1, which interacts with a hydrophobic pocket found only on this receptor (105). Interestingly, IgG2 contains a Val in that position, preceded by a Pro, which could explain its complete lack of binding to FcγRI. Comparisons between Fc-only and Fc:FcγR complex structures reveal that upon binding, the CH2 domains become stabilized while moving away from each other. C1q has a partially overlapping Fc binding site with FcγRs, although its binding is driven mostly by electrostatic interactions (106).

Another important determinant of the Fc:FcγR interaction strength is the *N*-linked glycan found at position N297 in the C′/E loop of the CH2 domain. Removing the core fucose from the IgG glycan increases affinity toward FcγRIIIa and FcγRIIIb (107) without significantly affecting the affinity of the Fc toward other FcγRs. This occurs primarily because the Fc fucose sterically clashes with the N162 glycans, present only on the FcγRIII receptors (108). Interchain glycan:glycan interactions between chains A and B are thought to stabilize the Fc, and their removal results in an 8°C drop in the melting temperature of the CH2 domain (109). In addition, removing the glycans results in near complete loss of binding to FcγRs, with the exception of FcγRI (27). This has been suggested to occur because in the absence of the glycan, the CH2 domains become more disordered, thus increasing the entropic cost for the Fc binding to FcγRs. While X-ray crystallographic and spectroscopic data support this hypothesis (110),

nuclear magnetic resonance studies have revealed similar CH2 orientations in glycosylated and aglycosylated Fc proteins, with only the C′/E loop of the CH2 domain displaying increased mobility upon glycan removal (111). Hence, it appears that the glycan specifically stabilizes the C′/E loop, which is necessary for FcγR binding. Indeed, the Fc D265A mutation that destabilizes the glycan's interaction with the protein backbone and by extension the C′/E loop results in poor binding to FcγRs, similarly to aglycosylated Fc (27).

The interface between the CH2 and CH3 domains includes the binding site for FcRn and TRIM21 and also for pathogen receptors linked to immune evasion, including the *Staphylococcus aureus* protein A, streptococcal protein G, and gp68 from human cytomegalovirus (112). Likewise, FcαRI binds to the homologous domain in IgA, between Cα2 and Cα3 (113), with one Fc molecule capable of engaging two receptor molecules at the same time. The CH2:CH3 interface consists of two α-helices at the bottom of the CH2 domain, three loops at the top of the CH3 domain, and the flexible linker between the two domains. The binding of FcRs that interact with the CH2:CH3 interface is independent of the Fc glycan.

The binding of IgG to FcRn has been studied in great detail. IgG binds to FcRn at the endosomal pH (pH < 6.0) but minimally binds at pH > 7.0. Binding to FcRn is mostly driven by hydrophobic interactions, hydrogen bonding, and salt bridges. There is one Fc His residue (side chain $pK_a \sim 6.0$) at position 310 that is directly involved in FcRn binding. This partly accounts for the pH-sensitive interaction (114). However, at least three more Fc His residues near the binding site (H429, H433, and H435) affect the pH-dependent binding to FcRn, although their precise contribution to the pH-dependent binding has not been fully resolved. Of note, hydrogen–deuterium exchange mass spectrometry (HDX-MS) revealed a destabilization of the CH2:CH3 interface upon pH change, which was required for FcRn binding, suggesting that internal reorganization of the Fc CH2:CH3 elbow region precedes binding (115).

Indeed, the CH2:CH3 interface displays increased mobility, as evidenced by molecular dynamics simulations (116). Salt bridges between the CH2 and CH3 domains are thought to stabilize the CH2 conformation and thus are relevant to FcRn binding but can also allosterically influence binding to distal receptors. Quite remarkably, several mutations at the CH2:CH3 interface have been shown to decrease affinity to the effector FcγRs, despite being located far from the FcγR binding site (117). Of relevance to protein therapeutics is the finding that mutations that increase the affinity for FcRn at acidic pH and mediate longer circulation persistence (see Section 5.3) partially impair effector FcγR binding (118). Conversely, mutations at the top CH3 domain of an aglycosylated IgG rescue binding to FcγRI (119) by stabilizing the conformation of the CH2 domain.

Finally, we note that there is evidence in support of the long-held assumption that antigen binding can allosterically modulate effector functions. For example, Orlandi et al. (120) observed increased FcγR engagement upon antigen binding. Surprisingly, HDX-MS analysis showed that, following antigen binding, conformational rearrangements were observed at the CH2:CH3 interface and not at the FcγR binding site. When bound on a target cell at high density, IgG Fcs can engage in low-affinity, homotypic interactions at the CH2:CH3 interface, resulting in the generation of hexameric oligomers on the cell surface, similar to IgM hexamers (121). In turn, this results in more efficient binding to the hexameric C1q, which, as mentioned above, requires at least four antibody molecules to initiate the complement cascade (122). Single mutations at the CH2:CH3 interface (E345R or E430G) increase oligomer assembly on surfaces. Due to the very low affinity of this Fc:Fc interaction, it is unclear if Fc conformational changes upon antigen binding are required to potentiate oligomerization, or if it is purely driven by the high local concentration of IgG on the surface.

Additionally, low-affinity, homotypic interactions outside the Fc domain can also be important for IgG effector functions. Homotypic antibody associations arising from weak Fab:Fab interactions that occur after antigen binding and stabilize the antibody:antigen complex have been known for many years. However, their significance for therapeutic antibody function with respect to inducing IgG clustering on the surface target cells was not appreciated until recently (123). Rougé et al. (124) showed that rituximab, although it binds CD20 with a 1:1 stoichiometry, engages in homotypic self-interactions via its variable domain with a neighboring rituximab:CD20 complex, thus promoting CD20 dimerization and enhancing CDC potency. Similar homotypic interactions have been observed with protective antibodies that recognize a repeat peptide sequence in the circumsporozoite protein of the malaria parasite *Plasmodium falciparum*.

5. Fc ENGINEERING TO MODULATE ANTIBODY FUNCTIONS

5.1. Altering Fc Affinity to Activating FcγRs

In early studies, structural information on Fc:FcγR interactions was utilized to generate variants with enhanced binding to the activating FcγRs. In a seminal study, Lazar et al. (125) generated IgG1 variants with increased affinity toward FcγRIIIa by mutating the lower hinge (S239D) and the F/G loop of CH2 (A330L and I332E). Interestingly, the affinity toward the inhibitory FcγRIIb was also increased, albeit to a lesser extent. Double or triple combination mutants display increased ADCC by peripheral blood mononuclear cells and ADCP by macrophages (125) (see tafasitamab in **Table 1**). Another lower-hinge mutation (G236A) selectively increases the FcγRIIa affinity over FcγRIIb, translating to better macrophage phagocytosis (39). Combining the FcγRIIa- and FcγRIIIa-enhancing mutations results in an Fc variant with higher affinity to activating FcγRs over the inhibitory FcγRIIb (high A/I ratio; see elipovimab in **Table 1**). In another example, Stavenhagen et al. (126) isolated more than 400 possible Fc mutations that enhanced FcγRIIIa and decreased FcγRIIb affinity by screening a random library of Fc variants displayed on the surface of yeast. By combining mutations on both the extended FcγR binding site (F243L, R292P, and Y300L) and the CH2:CH3 interface (P396L), they achieved a tenfold increase in FcγRIIIa affinity while maintaining relatively low FcγRIIb binding (see margetuximab in **Table 1**).

As mentioned above, antibody afucosylation results in increased affinity only to the FcγRIII receptors, a result of unique glycan (receptor):glycan (Fc) interactions (108). Due to the minimal impact on the biophysical antibody stability and the manufacturing process, afucosylated antibodies were quickly introduced into the clinic (see obinutuzumab in **Table 1**). Apart from superior NK cell–mediated killing, afucosylated antibodies result in better phagocytosis by monocytes and macrophages, despite the low level of FcγRIIIa expression on these cells (35). Conversely, the increased affinity to FcγRIIIb restricts neutrophil-mediated tumor cytotoxicity (93).

Apart from better cytotoxicity in vitro, these Fc variants have shown superior activity in various in vivo models. However, in vivo evaluation of Fc-engineered antibodies hinges on the use of representative animal models. Since the mouse and human *FCGR* gene families differ substantially, in vivo studies require the use of humanized mice expressing human FcγRs while carrying deletions of the mouse receptors (127, 128). For such studies it is important that the expression level and tissue distribution of human FcγR be representative of what are observed in humans. The evaluation of Fc-engineered antibodies in humanized FcγR models has yielded many key insights relevant to therapeutic antibody design. In one notable recent example, when tested in a mouse model of influenza, Fc mutants with increased affinity toward FcγRIIa (containing the G236A mutation), but not those with increased affinity toward FcγRIIIa (i.e., an afucosylated variant or a variant with the A330L/I332E substitution), showed improved protection from challenge

with a lethal dose of influenza virus (129). This protective effect was dependent on $CD8^+$ T cells and was attributed to the improved DC activation and antigen presentation. In another study by Ravetch and coworkers (130) using a tumor model, FcγRIIIa was shown to be necessary for tumor cytotoxicity, while FcγRIIa was critical for eliciting a robust adaptive T cell response.

Engineered Fc domains having extremely high selectivity toward a single effector FcR and negligible binding toward all others can be very informative for the mechanistic understanding of the phenotypes and cell signaling cascades mediated by Fc engagement of myeloid cells. Fc5 is the first engineered aglycosylated variant of IgG1 that does not bind to any of the low-affinity FcRs yet retains binding to FcγRI in a manner similar to that of unmodified IgG1 (119). It contains two mutations in the CH2:CH3 interface (E382V and M428I) and was shown to potentiate efficient killing of tumor cells by lipopolysaccharide-activated, monocyte-derived DCs. Likewise, Kang et al. (40) generated an aglycosylated Fc variant that engages only $FcγRIIIa_{V158}$. Antitumor antibodies with this Fc domain were shown to elicit efficient clearance of tumor cells via ADCP with granulocyte-macrophage CSF–differentiated macrophages as effectors, even though they express low levels of FcγRIIIa relative to the other FcγRs. Recent studies have shown that, in fact, FcγRIIIa on macrophages plays a key role in tumor clearance in vivo (130). Engineering a panel of highly selective Fc domains to each receptor provides an opportunity for elucidating the signaling and phenotypic differences resulting from the engagement of each FcγRI, FcγRIIa/b, and FcγRIIIa/FcγRIIIb.

For many therapeutic purposes in which antibodies are used as receptor agonists or antagonists, effector functions can be detrimental. For this reason, agonistic (or antagonistic) antibodies are often of the IgG2 or IgG4 subclass, which, as mentioned above, has minimal binding to most FcγRs to avoid depleting the cells expressing the cognate receptor by ADCC, ADCP, or CDC (26). However, IgG2 or IgG4 antibodies can still initiate effector functions, primarily by myeloid cells (131, 132), due to residual binding to FcγRIIa and/or FcγRI (133). Fc engineering has been employed to achieve complete effector silencing of blocking antibodies. Early studies showed that removal of the Fc N297 glycan, by mutating the glycosylation site (see atezolizumab in **Table 1**), attenuates but does not completely abolish ADCC and ADCP (134). From a manufacturing standpoint, aglycosylated antibodies can be expressed in alternative hosts, which can lower production costs, as seen in the case of eptinezumab (Vyepti®), which is made in *Pichia pastoris*. Alternatively, mutations in the lower hinge have been used to disrupt the conserved FcγR binding site, with the most notable example being the double mutant L234A/L235A (known as LALA). Importantly, both these formats maintain some residual FcγR binding, primarily to FcγRI under high avidity conditions (27, 135). Additional mutations in the FcγR binding interface, in combination with hinge mutations, are required for complete loss of FcγR engagement even at high avidity conditions (136).

Optimal agonistic activation of certain receptors, including members of the TNF receptor superfamily such as CD40 or TRAIL, is predicated on receptor clustering (9). Receptor clustering by agonistic antibodies can be aided by FcRs present on effector cells and is referred to as FcγR scaffolding. This scaffolding effect can be driven by all FcγRs (137); however, to circumvent cytotoxicity, it is desirable to reduce affinity toward activating receptors and instead only engage FcγRIIb. Indeed, selectively increasing affinity toward FcγRIIb (using the S267E mutation) was shown to result in greater tumor apoptosis by an anti-DR5 monoclonal (138). However, this study was performed in mice expressing only human FcγRIIb and not any of the activating FcγRs, since the S267E mutation does not abolish binding to the activating FcγRs, and thus toxicity effects could not be evaluated (139). To address this problem, Mimoto et al. (140) generated an IgG1 Fc variant, V12, that binds to FcγRIIb with very high selectivity over most activating FcγRs.

Anti-CD40 antibodies formatted with the V12 Fc were shown to be safe and effective in a humanized mouse model (141). However, while better than other FcγRIIb-selective Fcs, V12 still retains near wild-type binding to $FcγRIIa_{R131}$ and therefore could result in adverse effects in humans. Yet another approach to increase receptor agonism is to take advantage of the propensity of IgG Fcs to oligomerize by Fc:Fc interactions (121). Addition of the HexaBody® mutation (E430G) can increase the receptor clustering as well improve the recruitment of complement (see HexaBody-DR5/DR5 in **Table 1**). HexaBody antibodies retain binding to FcγRs and also have a similar pharmacokinetic profile to wild-type antibodies (142); thus, they provide an excellent platform for targets where agonistic activity and cytotoxicity are desired.

5.2. Enhancing Fc Domains for Higher Complement Activation

Complement provides another major pathway through which antibodies exert their cytotoxicity toward pathogens and viruses. The contribution of the complement cascade in tumor clearance varies, depending on tumor type, with the presence of complement inhibitors, antigen density, and even targeting epitope on the antigen being the key factors in regulating complement-dependent cell killing (143). Although CDC against various tumor cell lines is observed in vitro, the relative contribution of the complement pathway versus the FcγR effector pathway in tumor eradication remains unclear. The introduction of the K322A mutation in the lower CH2 domain greatly reduces binding to C1q and CDC while only slightly affecting FcγR binding. Loss of binding to C1q did not affect rituximab-mediated B cell clearance in a humanized mouse model (144). Conversely, Boross et al. (143) have shown that complement activation is sufficient to mediate the depletion of $CD20^+$ leukemic cells in $FcRγ^{-/-}$ mice (lacking all activating receptors), with C3b deposition contributing to cell-mediated killing (CDCC and/or CDCP), in parallel with CDC. In addition, using an engineered aglycosylated Fc that does not bind FcγRs, Lee et al. (55) showed that C1q binding alone induces complement-driven cytotoxicity and phagocytosis in vitro and suppresses tumor growth of $CD20^+$ cells in nude mice. Besides the hexamerization mutations, increasing the affinity toward C1q by mutating the Fc (S267E, H268F, and S324T) results in increased cytotoxicity and complement effectors (145). Importantly, these mutations can be combined with the FcγR-enhancing modifications to maximize cytotoxicity.

5.3. Engineering Binding to FcRn

Increasing the persistence of monoclonal antibodies in circulation reduces dosing requirements and increases drug bioavailability. Fc mutations increasing affinity to FcRn do not necessarily result in increased half-life in circulation (146). Instead, retaining the pH-sensitive binding to FcRn is critical for more efficient endosomal recycling and thus for extending antibody half-life (128). Many Fc variants with enhanced pharmacokinetics have been discovered, with mutants M252Y/S254T/T256E (147) and M428L/N434S (Xtend®; see ravulizumab in **Table 1**) (148) being shown to increase antibody half-life in humans by more than twofold.

Intravenous immunoglobulin (IVIg), pooled serum IgG from thousands of donors, is used to treat a plethora of autoimmune and hyperinflammatory syndromes. High doses of IVIg (usually >1 g/kg) compete with endogenous IgG for FcRn-mediated recycling, thus drastically decreasing autoantibody persistence in serum. An engineered Fc domain with increased affinity to FcRn at both pH 6.0 and 7.4 has been shown to interfere with the recycling of IgG antibodies, resulting in a significant reduction in the level of circulating pathogenic autoantibodies, and has shown therapeutic benefit in multiple autoimmune diseases including myasthenia gravis, thrombocytopenia, and others (see efgartigimod in **Table 1**) (149).

5.4. Other Fc Engineering Approaches for Antibody Therapeutics

IVIg can exert direct anti-inflammatory effects that are independent of FcRn. Sialylated IVIg has been reported to be more effective at lower doses (150). Although terminal sialylation slightly affects the affinity to FcγRs (151), the effect of the sialic acid is mainly ascribed to binding to type II FcRs, such as SIGN-R1 in mice or DC-SIGN and CD23 in humans. However, the exact mechanism by which these receptors mediate anti-inflammatory effects remains controversial (152). Nonetheless, ex vivo hypersialylated IVIg is currently being tested in a phase II clinical trial for ITP and chronic inflammatory demyelinating polyneuropathy (see M254 in **Table 1**).

Soluble, multimeric Fcs are capable of blocking FcγR-driven inflammatory reactions (153). Ortiz et al. (153) investigated the optimal valency and conformation requirements of multimers consisting of wild-type IgG1 Fcs and showed that Fc trimers blocked FcγR engagement without resulting in their activation and outperformed IVIg in preclinical arthritis and ITP mouse models (see CSL730 in **Table 1**). Fc engineering approaches that enhance binding to FcγRIIb have also been employed as autoimmune or allergy interventions (139, 140). The dual mutation S267E/L328F was shown to increase affinity to FcγRIIb (and $Fc\gamma RIIa_{R131}$) by 430-fold, blocking Ca^{2+} flux and promoting apoptosis in B cells, when formatted with an anti-CD19 (139) or an anti-IgE Fabs. Anti-IgE Xmab7195 selectively inhibits B cells expressing membrane-bound IgE while also exhibiting increased clearance of soluble IgE by LSECs. However, antibodies with enhanced FcγRIIb affinity are rapidly cleared from circulation by LSECs, which express high levels of FcγRIIb. As a result, antibodies formatted with the S267E/L328F mutations exhibit a serum half-life of only 3–4 days in humans. The increased clearance rate has been exploited to construct what have been termed sweeping antibodies that serve to rapidly clear target molecules from circulation. Sweeping antibodies incorporate three antibody engineering innovations: (*a*) a variable region engineered to allow dissociation of the antigen at endosomal pH, resulting in the release of the antigen into the endosomal lumen and subsequent degradation; (*b*) enhanced FcRn-mediated antibody recycling; and (*c*) high affinity to FcγRIIb, which leads to enhanced clearance of the antibody:antigen complex by LSECs, resulting in degradation of the antibody as well as the antigen (154). The sweeping antibody GYM329, which inhibits and also mediates clearance of myostatin, a member of the transforming growth factor-β superfamily, has been shown to increase muscle mass in animal models and is currently in clinical development (155).

For completion's sake, it should be mentioned that Fc engineering has also been used successfully to enable binding to receptors other than the canonical FcγRs, FcRn, and C1q. In a recent impressive study, Kariolis et al. (156) reported the engineering of a binding site to the transferrin receptor on the side surface of the IgG CH3 domain, without affecting pH-dependent FcRn binding. In a cynomolgus model, transferrin receptor binding resulted in a dramatic increase of antibodies that could cross the blood–brain barrier. Alternatively, by fusing the IgG and IgA Fc domains, Kelton et al. (157) created a cross-isotype antibody (termed IgGA) capable of binding to human activating FcγRs and C1q as well as FcαRI.

6. CONCLUDING REMARKS

Over the last 30 years, a combination of (*a*) advances in our understanding of the biology of FcRs together with (*b*) protein engineering of the IgG Fc domain using either structure-guided mutagenesis or random mutagenesis and screening to achieve desired binding profiles to therapeutically relevant receptors and (*c*) the use of humanized murine models and other novel tools for evaluating Fc effector functions has opened the way for the design of an array of important antibody therapeutics with improved, or in some cases novel, mechanisms of action. As of 2021, well more than 30 antibody therapeutics that are approved or in clinical development have engineered

Fcs, most often for enabling improved half-life. As is evident from the several examples presented in this review, Fc engineering is also proving very useful for a better mechanistic understanding of the subtle differences and phenotypic effects mediated by each of the effector FcRs expressed on myeloid cells. This information is likely to prove very relevant for further fine-tuning of IgG effector functions as needed for specific therapeutic applications. Given the modular nature of the Fc, combining mutations to maximize the therapeutic potency is possible. For example, elipovimab contains FcγR-enhancing mutations (G236A/S239D/A330L/I332E) as well as the Xtend mutations (M428L/N434S). Yet another particularly topical example is VIR 7832, a potent severe acute respiratory syndrome coronavirus 2 neutralizing antibody that incorporates the G236A/A330L/I332E substitutions for enhanced FcγRIIa and FcγRIIIa binding and improved effector functions together with the Xtend mutations. Careful design is required when combining different sets of mutations, since, despite the distance between FcγR and FcRn binding sites on the Fc, there is the possibility of allosteric regulation between the two domains. This effect has been documented in the diminished FcγR binding of Fcs carrying FcRn-enhancing mutations (118). Combining Fc engineering with high-throughput monitoring of effector functions can shed light on such subtle effects and perhaps help reveal new therapeutic modalities (158).

DISCLOSURE STATEMENT

G.G. is an inventor on patent applications on Fc-engineered variants.

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